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Production and Characterization of Chitin-Glucan Complex by *Komagataella pastoris*

Dissertação para obtenção do Grau de Doutor em Engenharia

Química e Bioquímica

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Resumo

O tema desta tese centrou-se na produção, extração e caraterização do complexo quitina-glucanos, denominado por CQG. Neste processo, utilizou-se como principal fonte de carbono um subproduto da indústria do biodiesel – o glicerol. A levedura *Komagataella pastoris* (anteriormente denominada por *Pichia pastoris*) foi selecionada para a produção de CGC devido ao facto de atingir elevadas densidades celulares, usando como fonte de carbono o glicerol proveniente da produção de biodiesel.

Numa primeira abordagem, foram realizados ensaios em Erlenmeyer, no sentido de selecionar a estirpe de *K. pastoris* com melhor desempenho, a nível de crescimento, usando o glicerol como fonte de carbono. A estirpe *K. pastoris* DSM 70877 atingiu elevadas densidades celulares (82-95 g/l), comparativamente à estirpe DSM 70382 (76-89 g/l) usando como fonte de carbono glicerol puro (99 %, w/v) ou o glicerol resultante da indústria do biodiesel (86 %, w/v), respetivamente. Tendo em conta os resultados obtidos nos testes em Erlenmeyer, a estirpe DSM 70877 foi selecionada e desta forma usada em ensaios em bioreator (2 l), usando como única fonte de carbono o glicerol resultante da indústria do biodiesel, a uma concentração de 40 g/l.

A produção de biomassa de *K. pastoris* em bioreator foi realizada em condições controladas de temperatura e pH (30.0 ºC e 5.0, respetivamente). Nestas condições, a concentração de biomassa atingiu valores superiores a 100 g/l, em menos de 48 horas. Durante a fase "*batch*", o rendimento de biomassa em função do glicerol consumido foi de 0.55 g/g enquanto que na "*fed-batch*" atingiu 0.63 g/g.

No sentido de otimizar a eficiência do processo de extração e purificação do CQG da biomassa de *K. pastoris* foram efetuados diversos testes. Os resultados demonstraram que o uso de NaOH 5 M, a 65 ºC, durante 2 horas, no processo de extração, seguido de neutralização com HCl e sucessivas lavagens com água para baixar a condutividade (≤20µS/cm), aumentava a pureza do polímero. O polímero obtido, CQG_{puro}, apresentava uma razão molar quitina:β-glucanos de 25:75 %mol, semelhante ao valor do CQG comercial (30:70 %mol) (kiOsmetine da Kitozyme), extraído do micélio de *Aspergillus niger*. O CQG_{puro} foi também caraterizado por espetroscopia de ressonância magnética nuclear (RMN) e por calorimetria diferencial de varrimento (DSC), revelando um polímero com grau de pureza mais elevado, comparativamente ao CQG comercial (kiOsmetine).

De modo a otimizar a produção de CQG, foi estudado o impacto da temperatura (20-40 ºC) e do pH (3.5-6.5) na taxa específica de crescimento, na produção de CQG e na composição do polímero. Para tal, foram utilizadas ferramentas estatísticas, tais como a metodologia de superfície de resposta e o desenho compósito central. Os resultados mostraram que o conteúdo CQG na biomassa e a produtividade volumétrica (r_p) não foram significativamente afetadas na gama de valores de pH e temperatura testadas. Contrariamente, o efeito do pH e da temperatura teve impacto positivo na proporção molar de CQG na biomassa. A maior razão molar quitina:β-glucanos (> 14:86) obteve-se na gama de pH de 4,5-5,8 e de temperaturas de 26-33 ºC. A capacidade de *K. pastoris* sintetizar CQG com variações na razão molar quitina:β-glucanos em função do pH e da temperatura é uma caraterística que pode ser explorada para obter polímeros com composição distinta adequados a diferentes aplicações.

Foi, ainda, avaliado o impacto de vários fatores na produção de CQG por *K. pastoris* e na composição da parede celular, nomeadamente na razão molar quitina:β-glucanos: a concentração de glicerol, o uso de diferentes fontes de carbono, a concentração de micronutrientes no meio, a presença de compostos inibitórios ou estimuladores no meio e o uso de resíduos e subprodutos de diferentes indústrias utilizados como fontes de carbono.

Os resultados obtidos mostraram que o aumento da concentração inicial de glicerol de 40 g/l para 60 g/l e da concentração de $CaSO_4$ de 6.8 mM para 200 mM estimulou o crescimento celular. Por outro lado, o aumento da concentração de $MgSO_4$. 7 H_2O de 60 mM para 140 mM não apresentou efeito significativo no crescimento celular, mas induziu o aumentou do conteúdo de CQG na biomassa (27%), comparativamente às condições padrão (15%). Verificou-se ainda que a presença de cafeína ou glucosamina, apesar de inibir o crescimento celular, aumentou a razão molar quitina:β-glucanos no polímero de CQG (19:81 e 23:77 %mol, respetivamente).

Relativamente ao uso de resíduos e subprodutos resultantes de diferentes indústrias (melaço de cana-de-açúcar, soro de leite e de borras de café), os resultados mostraram que a cultura de *K. pastoris* atingiu baixa concentração celular usando soro de leite, enquanto que o melaço de cana-de-açúcar e as borras de café induziram o crescimento celular e a produção CQG na biomassa. Estas últimas fontes de carbono foram testadas em bioreator. Os resultados mostraram que a concentração de biomassa obtida utilizando melaço de cana-de-açúcar e borras de café hidrolisadas (3,46 - 17,78 g/l, respetivamente) foi significativamente menor do que a obtida usando glicerol resultante da produção do biodiesel (33,6 g/l). A proporção de CGC na biomassa obtida com melaço de cana-de-açúcar atingiu 17,53%, o que corresponde a uma concentração CGC de 3,12 g/l.

Em suma, os resultados demonstraram que, para além do glicerol, *K. pastoris* é capaz de metabolizar diversas fontes de carbono, e que o CQG apresenta características semelhantes ao CQG comercial (kiOsmetine), tornando-o uma alternativa promissora para ser usado em várias aplicações industriais (cosmética, suplementos alimentares e saúde).

Palavras-Chave: Complexo quitina-glucanos, *Komagataella (Pichia) pastoris*, Resíduos/subprodutos industriais, Glicerol.

Abstract

This thesis was focused on the production, extraction and characterization of chitin:β-glucan complex (CGC). In this process, glycerol byproduct from the biodiesel industry was used as carbon source. The selected CGC producing yeast was *Komagataella pastoris* (formerly known as *Pichia pastoris*), due the fact that to achieved high cell densities using as carbon source glycerol from the biodiesel industry.

Firstly, a screening of *K. pastoris* strains was performed in shake flask assays, in order to select the strain of *K. pastoris* with better performance, in terms of growth, using glycerol as a carbon source. *K. pastoris* strain DSM 70877 achieved higher final cell densities (92-97 g/l), using pure glycerol (99%, w/v) and in glycerol from the biodiesel industry (86%, w/v), respectively, compared to DSM 70382 strain (74-82 g/l). Based on these shake flask assays results, the wild type DSM 70877 strain was selected to proceed for cultivation in a 2 l bioreactor, using glycerol byproduct (40 g/l), as sole carbon source.

Biomass production by *K. pastoris* was performed under controlled temperature and pH (30.0 ºC and 5.0, respectively). More than 100 g/l biomass was obtained in less than 48 h. The yield of biomass on a glycerol basis was 0.55 g/g during the batch phase and 0.63 g/g during the fed-batch phase.

In order to optimize the downstream process, by increasing extraction and purification efficiency of CGC from *K. pastoris* biomass, several assays were performed. It was found that extraction with 5 M NaOH at 65 ºC, during 2 hours, associated to neutralization with HCl, followed by successive washing steps with deionised water until conductivity of $\leq$20µS/cm, increased CGC purity. The obtained copolymer, CGC_{pure}, had a chitin:glucan molar ratio of 25:75 mol% close to commercial CGC samples extracted from *A. niger* mycelium, kiOsmetine from Kitozyme (30:70 mol%). CGC_{pure} was characterized by solid-state Nuclear Magnetic Resonance (NMR) spectroscopy and Differential Scanning Calorimetry (DCS), revealing a CGC with higher purity than a CGC commercial (kiOsmetine).

In order to optimize CGC production, a set of batch cultivation experiments was performed to evaluate the effect of pH (3.5–6.5) and temperature (20–40 ºC) on the specific cell growth rate, CGC production and polymer composition. Statistical tools (response surface methodology and central composite design) were used. The CGC content in the biomass and the volumetric productivity (r_p) were not significantly affected within the tested pH and temperature ranges. In

contrast, the effect of pH and temperature on the CGC molar ratio was more pronounced. The highest chitin: β-glucan molar ratio (> 14:86) was obtained for the mid-range pH (4.5-5.8) and temperatures (26–33 ºC). The ability of *K. pastoris* to synthesize CGC with different molar ratios as a function of pH and temperature is a feature that can be exploited to obtain tailored polymer compositions.

The influence of several parameters on *K. pastoris* growth and CGC production were evaluated: glycerol concentration, different carbon sources, micronutrients composition, the presence of toxic or stimulatory compounds and the use of wastes and byproducts resulted from different industries as carbon sources. Furthermore, the effect of these factors on chitin:β-glucan molar ratio in yeast cell wall composition was also evaluated in two sets of shake flask experiments.

It was found that increasing glycerol concentration from 40 to 60 g/l and $CaSO_4$ from 6.8 to 200 mM stimulated cell growth. In contrast, the increase of $MgSO_4$. 7 H_2O from 60 mM to 140 mM, had little effect on growth, but increased CGC content on the biomass (27%), compared to the standard conditions (15%). Furthermore, the presence of caffeine or glucosamine despite inhibit yeast cell growth, increased the chitin molar ratio in the CGC polymer (19:81 and 23:77 mol%, respectively).

The impact of using wastes and byproducts resulted from different industries (sugarcane molasses, cheese whey and spent coffee grounds) as carbon sources for *K. pastoris* cultivation and on CGC production was studied. The results showed that *K. pastoris* reached lower biomass concentration using cheese whey, compared to the other substrates. The latter were selected and tested in bioreactor. Results showed that biomass concentration obtained using sugarcane molasses and spent coffee ground hydrolysate (3.46 - 17.78 g/l, respectively) were much lower than the results obtained with glycerol biodiesel byproduct (33.6 g/l). Furthermore, a CGC content of 17.53 %, that corresponds a CGC concentration of 3.12 g/l, was achieved with sugarcane molasses.

Results demonstrated that, beyond glycerol, *K. pastoris* can use several carbon sources, and CGC can be obtained from biomass with characteristics similar those reported to CGC commercial (kiOsmetine), making it a promising alternative to be used in several industrial applications (cosmetic, food complement and health).

Keywords: Chitin-glucan complex, *Komagataella (Pichia) pastoris*, Industrial wastes/byproducts, Glycerol

Nomenclature

Abbreviations

Acetyl Co-A	Acetyl coenzyme A
AIM	Alkaline-insoluble material
ATP	Adenosine triphosphate
ANOVA	Analysis of Variance
AOX	Peroxidase alcohol oxidase
AOX1	Ethanol-regulated alcohol oxidase 1 gene
ASM	Alkaline-soluble material
Asn	Asparagine
BOD	Biochemical oxygen demand (mg/l)
BSM	Basal salts medium
CCRD	Central Composite Rotatable Design
CDW	Cell Dry Weight (g/l)
CGC	Chitin-Glucan Complex
COD	Chemical oxygen demand (mg/l)
DHAP	Dihydroxyacetone phosphate
DNA	Deoxyribonucleic acid
DSC	Differential scanning calorimetry
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulture
EFSA	European Food Safety Authority

FAD	Flavin adenine dinucleotide
Glc	Glucose
GlcN	Glucosamine
GlcNAc	N-acetyl-D-glucosamine
HCl	Hydrochloric acid
HPLC	High Performance Liquid Chromatography
Man	Mannose
MLR	Multiple Linear Regression
NADH	Nicotinamide adenine dinucleotide
NaOH	Sodium Hydroxide
NMR	Nuclear magnectic ressonance
PBS	Phosphate-buffered saline solution
PTM	Trace salts medium
rpm	Rotation per minute
RSM	Response Surface Methodology
SCG	Spent Coffee Grounds
TCA	Tricarboxylic acid
TFA	Trifluoroacetic acid
UDP	Uridine diphosphate
vvm	Gas volume per liquid volume per time
WCW	Wet cell weight (g/l)

Variables

Abs (750 nm)	Absorbance at 750 nm
h	Hours
KDa	Kilodalton
N	Normal concentration
mol %	Molar Percentage
M	Molar concentration (mol/l)
M_r	Relative molecular mass
pO2 (%)	Dissolved Oxygen Concentration
ppm	parts per million
p-value	Regression parameter significance
R^2	Multiple correlation coefficient
r_p	product volumetric productivity (gCGC/l.h)
r_x	Biomass Volumetric Productivity (g/l.h)
T	Temperature (^oC)
μ_{max}	Maximum specific growth rate (h^{-1})
wt.%	Weight percentage

Greek Letters

α	Axial level in RSM
δ	Chemical shift

μ	Specific growth rate (h^{-1})
μS	Microsiemens

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Chapter 1

Background and Motivation

Industrial biotechnology is seen as one of the key technologies of the new age. It is mainly based on biological and engineering sciences and it develops new methods for the production of various important products for human health and the environment. Biotechnology not only helps in producing important biomaterials, usually non-toxic and biodegradable, such as polysaccharides, but it also makes possible to manufacture new products or value-added products, which were once thought to be impossible. In particular, the demand for novel polysaccharides with interesting properties for industrial applications (e.g. cosmetic, feed, medicine and pharmaceuticals) has greatly increased (Smirnou et al., 2011; Meichik and Vorob'ev, 2012). This thesis aimed at optimizing a process (previously described on patent WO2010/013174) to produce and purify chitin:β-glucan complex (CGC) from *Komagataella pastoris* (previously known as *Pichia pastoris*) cell wall biomass using glycerol byproduct from the biodiesel industry.

1.1.The yeast cell wall

Yeast cells are surrounded by a tough, rigid cell wall that represents 20-25% of the dry weight of the cell (Fleet, 1991; Klis et al., 1994). The major components of yeast cell walls are polysaccharides (85-90%) and proteins (5-15%). However, the cell wall ratio of each component varies with the species and with the biochemical adaptation to adverse factors (Feofilova et al., 2006). Most of the protein is covalently linked to mannan polysaccharides, forming mannoproteins.

The polysaccharide component consists of a mixture of water-soluble mannans, alkali-soluble glucans, alkali-insoluble glucans and small amounts of chitin (Table 1.1). Fleet and Manners (1976) showed that in *S. cerevisiae* there are approximately equal proportions of mannans and glucans and about equal amounts of alkali-soluble glucans and alkali-insoluble glucans.

Table 1.1 - Macromolecules of the cell wall of *S. cerevisiae* (Klis et al., 2005).

Macromolecule	**% of wall mass**	**Mean *M*r (DP) (kDa)**
Mannoproteins[a]	30–50	Highly variable
1,6-*β*-Glucans	5–10	24 (150)
1,3-*β*-Glucans	30–45	240 (1500)
Chitin	1.5–6	25 (120)

[a] The actual protein content is about 4–5%; the remaining mass is from protein-linked, mannose-containing carbohydrate side-chains.

The presence of polysaccharides conferred the structural function in the yeast cell wall, whereas the mannoproteins are important for the cell wall permeability (Zlotnic et al., 1984; De Nobel et al., 2000). The inner layer of the cell wall of yeast and fungi is mostly formed by β-(1,3) and β-(1,6)-glucans (polymers of glucose residues joined, respectively, by β-(1,3) and β-(1.6) linkages) and chitin (Smirnou et al., 2011).

Chitin is a polymer formed by long linear homopolymer chains of β-(1,4)-linked N-acetylglucosamine (Kollar et al., 1995 and 1997) synthesized by various organisms, being a common component of the exoskeletons of invertebrates and the cell wall of fungi and yeasts (Synowiecki and Al-Khateeb, 2003; Dutta et al., 2004). The chitin content differs among different species of yeast, fungi and crustacean (Table 1.2).

Table 1.2 - Comparison of chitin content in different species.

Organism	Chitin Content (%)	References
Crustacean	13-40	Synowiecki and Al-Khateeb, 2003
Aspergillus niger	38-41	Feofilova et al., 2006
Saccharomyces cerevisiae	3.11 – 3.61	Fleet et al., 1991
Pichia pastoris	7.0	Roca et al., 2012

Chitin is considered to be responsible for an enormous tensile strength and significantly contributes to the overall integrity of the cell wall of yeasts and fungi, being covalently linked to β-(1,3)-glucans, a polymer of glucose units, forming co-polymers known as chitin-glucan complex (CGC).

The outer layer of yeast and fungi cell wall contains mainly mannans, polysaccharides that are mostly covalently linked to the cell wall proteins (glycoproteins) (Osumi, 1998).

1.2. Glucans and Mannans

Glucans are the major structural polysaccharides of the fungal and yeast cell walls, representing around 50–60% of the wall by dry weight, and are composed of repeating glucose residues that are cross-linked into chains through a variety of chemical linkages. β-(1,3)-glucans are the dominant glucans, but other glucans, such as β-(1,6)-, mixed β –(1,3)- and β-(1,4)-, α-(1,3)-, and α-(1,4)-linked glucans are also commonly found (Fleet et al., 1991; Kapteyn et al., 1999).

Glucans are synthesized by enzyme complexes associated with the plasma membrane and then are branched by cross-linked to chitin and mannoproteins to provide the cell wall with mechanical strength and integrity (Kollar et al., 1997).

Mannans are chains of up to several hundred mannose monomers that are added to yeast proteins via N- or O-linkages and named mannoproteins. Phosphorylation of the mannosyl side chains supply at surface of yeast cell walls a negative charge (Lipke and Ovalle, 1998). The mannoprotein demonstrated good emulsifying and stabilizing properties (Araújo et al., 2014).

With regard to solubility, β-(1,3)-glucans with significant number of links β-(1,6) in the branches, constituted a cell wall fraction soluble in alkaline medium, β-(1,6)-glucans, a soluble fraction in acidic medium, and also β-(1,3) glucan linked to chitin represented a fraction insoluble in acidic and basic media (Phaff, 2001).

β-glucans have revitalizing properties, act as anti-inflammatory agents, protect against UV radiation and have soothing, immunostimulating, anti-ageing, anti-wrinkle and antiacne effects (Pillai et al., 2005).

1.3. Chitin-Glucan Complex (CGC)

The chitin-glucan complex (CGC) is the main component of cell wall in yeasts and fungi, conferring stability and rigidity to the cells (Roca et al., 2012). Its molecular structure consists of chitin, a polymer of N-acetylglucosamine units, covalently linked to β-(1,3)-glucans, a polymer of glucose units (Figure 1.1). It can be extracted from the cell-wall of yeasts and fungi (Aguilar-Uscanga et al., 2003; Pestov et al., 2009; Smirnou et al., 2011; Tarabukina et al., 2005). The glucan and chitin cell wall biosynthesis occurs on the plasma membrane and the polymer chains are extruded into the cell wall space during their synthesis (Bowman and Free, 2006).

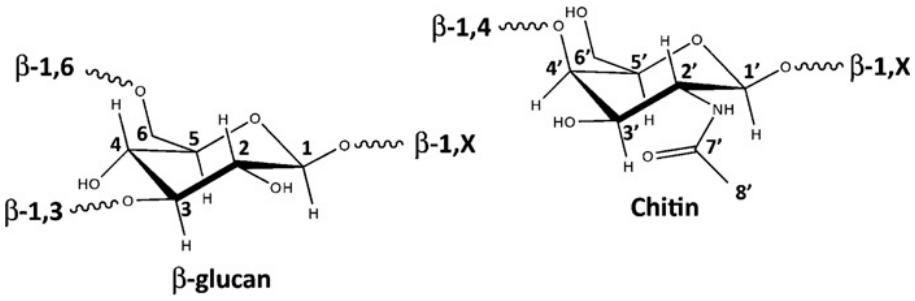

Figure 1.1 - Schematic representation of the molecular structure of β-glucan and chitin (Roca et al., 2012).

CGC is a biologically active and nontoxic co-polymer that has recently started to emerge as a valuable biomaterial for applications, such as cosmetics, agriculture, environment and medicine, and also as a source of non-animal chitin (Gautier et al., 2008).

Commercialization of CGC isolated from mycelia of the mold fungus *Aspergillus niger* has recently been authorized as food complement (EFSA, 2010). Further hydrolysis of CGC can easily lead to the production of many derivatives, such as pure chitin, chitosan (the deacetylated form of chitin) and glucans, which have attracted increasing interest due to their broad applications in agriculture, wastewater treatment, cosmetics or even in tissue engineering and immunology (Park et al., 2010; Rinaudo et al., 2006; Gautier et al., 2008). In addition, the production of fungal CGC offers the possibility to obtain a product with stable composition and properties, contrary to conventional preparation of chitin from marine sources.

Yeast production of CGC allows for the use of inexpensive raw materials, such as crude glycerol from the biodiesel industry (currently with no availability restrictions), and reaching high cell density process. Moreover, both the composition and the properties of the polymers are more stable than the ones obtained by the traditional extraction method from crustacean (Khanafari et al., 2008; Çelik et al., 2008).

CGC is extracted from yeasts and fungal biomass as an insoluble residue after successive treatments with alkali and/or acid, enzymatic methods, with the use of inorganic reagents, organic solvents or detergents (Ivshina et al., 2007; Sugawara et al., 2004). It can also be extracted from a by-product of the production of pharmaceutical and food-grade citric acid (Versali et al., 2003; Muzzarelli et al., 1997). Feofilova et al. (2006) reported a CGC content of 15-25% for fungi Aspergillus *niger* cell wall, and 25% for yeast, such as *S. cerevisiae.* As any other yeast cells, except *Schizosaccharomyces* species that do not contain chitin in the cell wall (Roncero and Durán, 1985), CGC is present in *K. pastoris* cells wall to maintain its integrity and, therefore, relatively high amount of CGC can be extracted after biomass production.

1.4. *Komagataella pastoris*

Pichia pastoris (reclassified as *Komagataella pastoris* (Yamada et al., 1995)) is a methylotrophic yeast able to metabolize reduced one-carbon compounds, such as methanol, using them as the sole carbon and energy sources for growth (Cunha et al., 2004).

Phillips Petroleum Company (USA) was the first company that developed cultivation techniques for *P. pastoris* species in large-volume continuous cultures and at cell densities higher than 130 g/l (Jahic et al., 2002) to produce single-cell protein for feed stock.

An advantage of *K. pastoris* over other yeasts is its capacity to use a wide range of substrates, including low-cost raw materials (e.g. biodiesel waste glycerol) (Bai et al., 2009; Cui and Ellison, 2012). Combining this feature with the fast growth rates (and hence shorter culture times), cheap operating costs and simple chemically defined media, make the production process economically viable and sustainable (Roca et al., 2012; Çelik et al., 2008).

In order to achieve maximal levels of the desired product with *K. pastoris* strains, it is important to understand the influence of culture conditions on the physiology and on the regulation of CGC production process. According to the literature, factors such as temperature (Li et al., 2001; Li et al., 2003; Shi et al., 2003), pH (Sreekrishna et al., 1997; Inan et al., 1999), composition of the feed medium (Boze et al., 2001; Xie et al., 2005; Jungo et al., 2007) and specific growth rate (D'Anjou and Daugulis, 2001) have to be optimized in order to achieve high productivities of desired products with *K. pastoris* strains.

1.5. Optimization of CGC Production

Available cultivation protocols for growing of *K. pastoris* are mainly based on those described in the Pichia Fermentation Process Guidelines (Supplied by Invitrogen). However, these standard protocols were developed to obtain heterologous proteins and not specifically to reach high cell density nor CGC.
At the moment, more than 500 heterologous proteins have been expressed by *P. pastoris* (Cregg et al., 2000 and Cos et al., 2006). Moreover, a recombinant DNA hepatitis B vaccine and interferon alpha derived from *P. pastoris* have been marketed in India (www.shantabiotech.com). Similarly, a recombinant human insulin was approved and marketed in India (www.biocon.com).

Efficient production of CGC relies on both the strain used and the optimal bioprocess parameters, such as carbon source, composition of the fermentation medium and operational conditions, such as temperature and pH. Although the effect of nutritional and environmental factors, such as temperature and pH, on growth and protein expression by *K. pastoris* have been extensively studied (Soyaslan and Çalik, 2011; Çalik et al., 2010; Cos et al., 2006; Gasser et al., 2007; Chiruvolu et al., 1998; Files et al., 2001; Inan et al. 1999 and Shi et al., 2003), their impact on this yeast cell wall composition, namely, on CGC content and chitin:β-glucan ratio, was not assessed.

1.5.1. Carbon Source

Glycerol

Biodiesel production generates about 10% (w/w) crude glycerol as the main byproduct. On the other hand, its cost is much lower than pure glycerol and is considered nowadays a surplus of this industry (Pachauri et al., 2006).

With the rapid growth of biodiesel industry all over the world, a large surplus of glycerol has been created and its price is declining day by day. The current market value is US$ 0.27- 0.41 per pound of pure glycerol (Yang et al., 2012) and as low as US$ 0.04 - 0.09 per pound of crude glycerol (Sims, 2011). Thus, crude glycerol disposal and utilization has become a serious issue and a financial and environmental liability for the biodiesel industry. Economic utilizations of glycerol for value-added products are critically important for the sustainability of biodiesel industry (Ayoub and Abdullah, 2012).

The metabolic network presented in Figure 1.2 is a macrokinetic model for reaching high cell densities of *K. pastoris* using glycerol from biodiesel industry as sole carbon source.

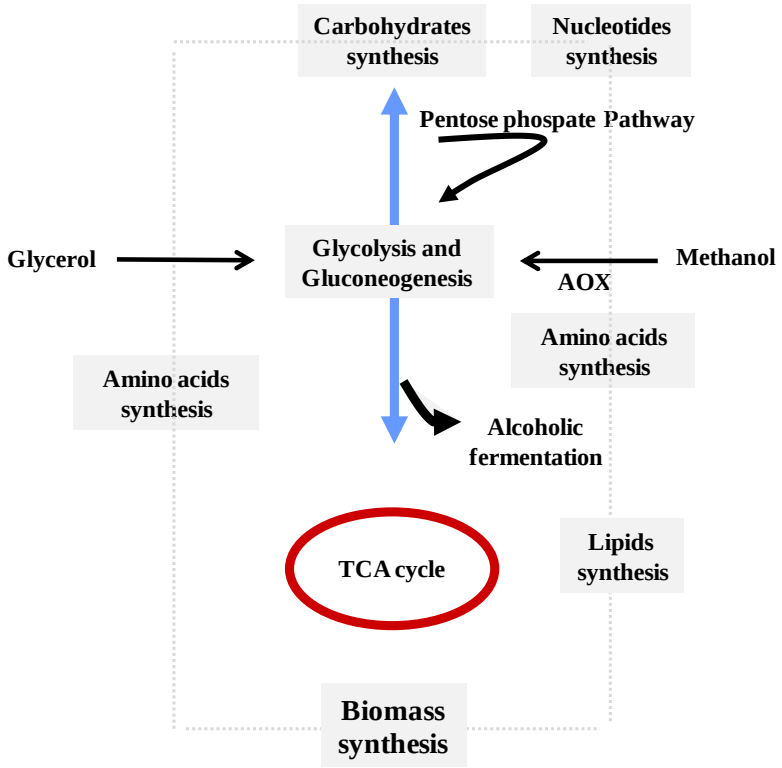

Figure 1.2 - Macrokinetic model for methylotrophic *K. pastoris* growth on glycerol byproduct from biodiesel industry.

The catabolic pathway includes glycerol phosphorylation by a glycerol kinase, resulting in the formation of glycerol 3-phosphate, which is then followed by oxidation to dihydroxyacetone phosphate (DHAP) by a FAD dependent glycerol-3-phosphate dehydrogenase. The dihydroxyacetone phosphate formed enters the glycolytic pathway. A few yeast species have an alternative pathway for dissimilating glycerol which involves a NAD-dependent glycerol dehydrogenase and a dihydroxyacetone kinase (Nevoigt and Stahl, 1997). Pyruvate is formed as an outcome of glycolysis and is further oxidized to acetyl-CoA, via pyruvate dehydrogenase. Acetyl-CoA enters the tricarboxylic acid (TCA) cycle, where many metabolites are produced and used for the synthesis of cellular constituents such as amino acids, nucleic acids and cell wall components (Ren et al., 2003; Solá et al., 2004).

1.6. Motivation

CGC is the main component of cell wall in yeasts and fungi, conferring stability and rigidity to the cells. Currently, commercial CGC is mainly obtained from *Aspergillus niger* from the citric acid production (Versali *et al.*, 2003) as a side-product of an industrial cultivation process. Nevertheless, those cultures do not attain cell densities as high as the values reached with yeasts, due the fact that the process is guided for the production of citric acid and not to production of high biomass densities and also due fungal morphology. As any other yeast cells, CGC is present in *K. pastoris* cell wall to maintain its integrity and, therefore, relatively high amount of CGC can be extracted after biomass production. This, combined with the fast growth rates, cheap operating costs, also due its ability to use efficiently inexpensive raw material and simple chemically defined media, make the production process economically viable and sustainable.

This PhD work plan aimed at optimizing the process described on patent WO2010/013174, whose objective was to produce high cell densities of *K. pastoris* to obtain chitinous polymers, as well as polymers containing glucose, mannose and/or galactose.

Bearing in mind that renewable resources, like industrial wastes/byproducts, may be regarded as potential alternative substrate sources for biopolymers production, in this PhD thesis, a process was studied to valorize glycerol byproduct from the biodiesel industry, as well other wastes/byproducts as carbon sources. For the first time, in this study, *K. pastoris* was grown on glycerol byproduct as carbon source at high cell densities to directly extract CGC from the cell biomass.

This thesis encompasses the following objectives:

Objectives

1. Screening of the most suitable *Komagataella (Pichia) pastoris* strains for cultivation at high cell densities, using the byproduct of biodiesel industry rich in glycerol as the sole carbon source.

2. Development and optimization of the extraction procedure to obtain pure CGC from *K. pastoris* biomass. Polymer characterization, in terms of its chemical composition; Thermal properties (DSC); elemental analysis; and NMR characterization to understanding the molecular-scale structure and dynamics of macromolecules contents on CGC polymer.

3. Optimization of bioreactor operation aiming at maximizing CGC production, by studding the effect of environmental conditions (pH and temperature).

4. Evaluation of the effect of medium composition and the use of stimulatory or inhibitory factors on the CGC production and on the impact on chitin:β-glucan molar ratio.

1.7. Thesis outline

This thesis is divided into six chapters, describing the work performed during this PhD project. The methodology used in each individual chapter is detailed in the context of the respective subject and, when applicable, is related to that used in previous chapters. Chapters 2 and 3 are dedicated to optimization of growth and CGC extraction and purification process optimization, as well as CGC characterization. Chapter 4 and 5 are related to optimization of the environmental conditions (pH and temperature), type and concentration of carbon source, presence of inhibitory/stimulatory factors and divalent ions, aiming at maximizing CGC productivity.
The work performed during this PhD resulted in three scientific papers, which have been published in international scientific publications.

Chapter 1: Background and Motivation
Chapter 1 introduces the subject of this thesis, by presenting the context and motivation for this PhD work.

Chapter 2: Screening of *Komagataella pastoris* strains

The second chapter of the thesis deals with the screening of high cell density producing *K. pastoris* strains. Two strains of *K. pastoris* with accession numbers DSM 70877 and DSM 70382 were tested. They were further grown in shake flasks using Basalt Salt Medium supplemented with pure and glycerol byproduct of biodiesel industry. Growth experiments on different carbon sources have been discussed here. Further, fed-batch culture was performed with strain *K. pastoris* DSM 70877 using glycerol from the biodiesel industry.

Chapter 3: Downstream Optimization and Polymer Characterization

Chapter 3 describes the studies performed for the optimization of extraction and purification of CGC. Various methods of extraction of CGC were tested aiming at making easier downstream processing after fermentation. Selective extraction by fractionation of *K. pastoris* biomass was assayed. The fractions extracted had low degree of purity, thus requiring further extraction and purification steps. Several assays were performed to increase extraction efficiency (extension of the reaction time, concentration of the alkali and temperature and purification of CGC (different solvent washing procedures).

Chemical and structural characterization of CGC was done by hydrolysis and analysis Dionex (HPLC), using elemental analysis, nuclear magnetic resonance (^{13}C NMR) and DSC.

Chapter 4: Effect of pH and temperature on CGC production

Chapter 4 focused on the interactive effect of temperature and pH using multivariate statistical analysis.
Response surface methodology (RSM) has been used for the optimization of bioreactor operation, by studding the effect of environmental conditions (pH and temperature).
This methodology has been extended to assess the cultivation conditions favorable for maximizing CGC production in yeast cell and evaluate chitin:β-glucan molar ratio in the polymer.

Chapter 5: Effect of Medium Composition on CGC Production

In chapter five, the effect of the type (namely, lactose, sucrose and galactose) and concentration of carbon source, presence of inhibitory/stimulatory factors (presence of glucosamine or caffeine) and increased concentration of some divalent ions (namely, Ca^{2+}, Mn^{2+} and Mg^{2+}) on CGC productivity and on chitin:β-glucans molar ratio were evaluated in shake flask assays. The use of food industry byproducts, namely, cheese whey, sugarcane molasses and spent coffee grounds hydrolysate, was also evaluated.

Chapter 6: Conclusions and Future strategies

The main conclusions obtained in this PhD thesis are presented in chapter 6. Some suggestions for future research are also proposed.

Chapter 2

Screening of Komagataella pastoris strains

2.1 Summary

This study aimed at selecting the most suitable *Komagataella (Pichia) pastoris* strain for cultivation at high cell densities, using the byproduct of biodiesel industry rich in glycerol as the sole carbon source.

Two strains, namely *K. pastoris* DSM 70877 and DSM 70382, were cultivated in pure glycerol (99%, w/v) and in glycerol from the biodiesel industry (86%, w/v), in shake flask assays. It was found that both strains grew well in either substrate. Nevertheless, strain DSM 70877 has achieved higher final cell densities in both experiments (82-95 g/l, respectively), compared to DSM 70382 strain (76-89 g/l, respectively). Based on these results, the wild type DSM 70877 strain was selected to proceed for cultivation in a 2 l bioreactor, using glycerol byproduct (40 g/l), as sole carbon source.

Glycerol from the biodiesel industry was used as carbon source for high cell density fed-batch cultivation of *Komagataella pastoris* DSM 70877 aiming a producing of biomass at high cell densities. More than 100 g/l biomass was obtained in less than 48 h. The yield of biomass on a glycerol basis was 0.55 g/g during the batch phase and 0.63 g/g during the fed-batch phase.

2.2 Introduction

Komagataella pastoris is a methylotrophic yeast. This ability to metabolize reduced one-carbon compounds, such as methanol, using them as the sole carbon and energy sources for cell growth, is possible due to overexpression of the enzyme peroxisome alcohol oxidase (AOX), which can reach about 30% of the intracellular protein in the presence of methanol (Couderc, 1980).

The prevalent use of *K. pastoris* in biotechnology is based on several advantages. One advantage consists in achieving high cell densities during fermentation, over 130 g/l cell dry weight, in controlled environmental conditions (Cregg et al., 1993; Cereghino and Cregg, 2000). Another advantage of *K. pastoris* is its capacity to efficiently use a wide range of substrates, including low-cost raw materials (e.g. biodiesel waste glycerol and methanol) (Çelik et al., 2008; Cui and Ellison, 2012), making the production processes more economically viable and sustainable (Roca et al., 2012; Reis et al., 2011; Ferreira et al., 2012; Çelik et al., 2008).

Glycerol, an abundant byproduct from the biodiesel industry, is today one of those cheap raw materials. In Europe alone, the biodiesel production was over 9 million tones in 2014 (www.longhini.eu). For every 9 kg of biodiesel produced, approximately 1 kg glycerol is obtained. Large amounts of glycerol are then expected to accumulate with increasing demand for biodiesel. However, glycerol resulting from biodiesel industry contains impurities, which makes it inadequate for use in many of the traditional glycerol applications unless costly purification steps are performed. For this reason, there is an urgent need to develop new ways to convert this glycerol byproduct, into higher value-added products. Furthermore, Çelik et al. (2008) already studied the influence of the type and purity/concentration of crude glycerol on *K. pastoris* growth and showed that cell concentration was higher using a medium containing crude glycerol (with a methanol content of >3%), as carbon source (reaching a biomass yield of 0.57 g biomass/g substrate), whereas lower glycerol consumption rates were obtained with pure glycerol medium, underlying the positive effect of potential additional nutrients present in crude glycerol.

K. pastoris is used to produce heterologous proteins at high levels when grown on a minimal medium. This organism is one of the most promising choices for future expression of human proteins for medical applications (Ferreira et al., 2012). The ability to reach high cell densities and to produce target products are due to: (a) the presence of regulated promoter from the methanol-regulated alcohol oxidase I gene (AOX1), (b) the efficient protein secretion system,

which simplifies protein purification, and (c) *K. pastoris* preference to grow in a respiratory mode, reducing excretion of fermentation byproducts, such as ethanol or acetic acid, often growth inhibitors, and allows one to reach exceptionally high cell densities (Solá et al., 2004).

The protocol for aerobic growth of *Komagataella pastoris* yeast to achieve high cell densities and protein production is well optimized (Oliveira et al., 2005; Ferreira et al., 2012). The components of *K. pastoris* media (glycerol, methanol, salts and trace elements) are relatively inexpensive and as such are well suited for large-scale production. As described by Cunha et al. (2004), *K. pastoris* fermentation usually occurs in semi-continuous mode, starting with a batch phase in a medium containing glycerol as the primary carbon source, followed by a stage of feeding with glycerol for biomass growth, and a final feed phase with a methanol stream as a carbon source for the production of the target protein. A high cell density is a key characteristic of *Komagataella pastoris* due to its preference for growth. This trait lends an advantage to *K. pastoris* over other yeasts, such as *Saccharomyces cerevisiae*, whose growth is hindered by the ethanol accumulated during fermentation.

The main objective of this chapter was the screening for a *K. pastoris* strain able to reach high cell densities in a medium containing glycerol from the biodiesel industry as the sole carbon source.

2.3 Material and Methods

2.3.1. Yeast strains and culture medium

The strains used in this work were purchased at the Leibniz Institute DSMZ – German collection of microorganisms and cell cultures. Two *Komagataella pastoris* strains were tested, namely DSM 70877 and DSM 70382. Both strains were cryopreserved in 20% (v/v) glycerol, at -80ºC.

The strains were cultivated in standard basal salts medium (BSM) (Pichia Fermentation Process Guidelines, Invitrogen), with the following composition (per litre): H_3PO_4 85%, 26.7 ml; $CaSO_4$, 0.93 g; K_2SO_4, 18.2 g; $MgSO_4{\cdot}7H_2O$, 14.9 g; KOH, 4.13 g; glycerol, 40 g; Antifoam A (Sigma), 0.75 ml and 4.35 ml of a trace elements solutions (PTM). The PTM solution had the following composition (per litre): $CuSO_4{\cdot}5H_2O$, 6 g; NaI, 0.08 g; $MnSO_4{\cdot}H_2O$, 3 g; $Na_2MoO_4{\cdot}2H_2O$, 0.2 g; H_3BO_3, 0.02 g; $CoCl_2{\cdot}6H_2O$, 0.5 g; $ZnCl_2$, 20 g; $Fe_2SO_4{\cdot}7H_2O$, 65 g; biotin, 0.2 g and H_2SO_4, 5 ml. The pH was adjusted to 5.0 with a 25% (v/v) ammonium hydroxide solution (Scharlau). The PTM solution was filter sterilized (0.2 μm, Sartorius Stedim Minisart) separately and added to the BSM medium after its sterilization at 121 ºC, for 30 minutes. Pure glycerol (Sigma-Aldrich, 99%) or glycerol byproduct (with a glycerol content ca. 86 w/v, supplied by SGC Energia, SGPS, SA, Portugal) were sterilized at 121 ºC, for 30 min.

BSM medium was used for inocula preparation, as well as for the shake flask and bioreactor experiments.

2.3.2. Shake flask tests

K. pastoris DSM 70877 and DSM 70382 were cultivated in BSM medium supplemented with 40 g/l pure glycerol (Sigma-Aldrich, 99%) (run E0) and glycerol byproduct at different concentrations, 43 and 50 g/l (runs E1 and E2, respectively).

Pre-inocula for the experiments were prepared in 125 ml shake flasks by inoculating 1 ml of the cryopreserved culture (stored at -80 ºC) in 40 ml of BSM medium, supplemented with pure glycerol (40 g/l). The pre-inocula were incubated in an orbital shaker for 48 hours, at 30 ºC and 200 rpm. Inocula for the experiments were prepared by inoculating 10% (v/v) of the pre-

inoculum (25 ml) in 250 ml BSM medium and further incubating the cultures, under the same conditions, for 72 hours.

For runs E0, E1 and E2, pre-inocula (25 ml) was used to inoculate a 500 ml shake flask with 250 ml BSM medium at 10% (v/v) and incubated in an orbital shaker at 30 ºC and 200 rpm, for 120 h. All experiments were performed in duplicate. Twice daily, 1 ml samples were withdrawn from each shake flask for measurement of the pH and the optical density at 600 nm.

2.3.3. Bioreactor operation

Inoculi for bioreactor experiments were prepared by incubating the culture in BSM medium, containing glycerol byproduct (40 g/l), in shake flasks for 2 days at 30 ºC, in an incubator shaker (250 rpm). This pre-inoculum was used to inoculate a 250 ml shake flask at 10% (v/v), which was grown for 3 days at 30 ºC and 250 rpm. Experiments were carried out in a 2 l bioreactor (BioStat B-plus, Sartorius) with an initial working volume of 1.4 l. The bioreactor was operated with controlled temperature and pH of 30 ºC ± 0.1 and 5.0 ± 0.05, respectively. pH was controlled by the addition of 25% (v/v) ammonium hydroxide that served also as the nitrogen source. The DO concentration was controlled by the automatic variation of the stirring rate (between 300 and 1000 rpm) and supplementation of the air stream with pure oxygen. An initial batch phase was performed during 26 h on BSM medium. Fed-batch mode was initiated when a decrease in the oxygen consumption rate was observed, by supplying the bioreactor with fresh glycerol byproduct (~86%, w/v) supplemented with 24 ml of trace elements PTM solution per liter of glycerol, using an exponential feeding rate, $F = F0e^{\mu t}$, with F being the feeding rate in g/h, F0 the initial feeding rate (5.6 g/h), and μ the desired specific growth rate, 0.16 h^{-1}.

2.3.4. Analytical Techniques

Culture broth samples were taken periodically from the bioreactor during the cultivation runs and centrifuged at 10 000×*g*, for 15 minutes, for cell separation. The cell-free supernatant was stored at -20 ºC for glycerol quantification.

The cell pellet was used for the gravimetric determination of the wet cell weight (WDW, g/l), after washing with deionised water (ressuspension in water, centrifugation at 10 000×*g*, for 15

minutes). The cell dry weight (CDW, g/l) was also determined by gravimetry, by drying the wet cell pellet at 105 ºC until constant weight.

Glycerol concentration in the cell-free supernatant was determined by high performance liquid chromatography (HPLC) with an Aminex HPX-87H column (BioRad), coupled to a refractometer. The cell-free supernatant samples were diluted in H_2SO_4 0.01 N and filtered with Vectra Spin Micro Polysulphone filters (0.2 μm), at 10 000×*g*, for 10 min. The analysis was performed at 50 °C, with sulphuric acid (H_2SO4 0.01N) as eluent, at a flow rate of 0.6 ml/min. An external standard calibration curve was constructed using glycerol (Sigma – Aldrich 99%) solutions in concentrations within 10 and 1000 ppm.

2.3.5. Kinetic parameters

The maximum specific cell growth rate (μ_{max}, h^{-1}) was obtained by linear regression of the logarithm of cell dry weight in relation to time, using the following equation:

$$Ln\left(\frac{CDW}{CDWo}\right) = \mu_{\max} t \qquad (1)$$

where CDW_0 (g/l) is the cell dry weight at the beginning of the run.

The yield of biomass on substrate ($Y_{X/s}$, g/g) was determined by using the following equation:

$$Yx/s = \Delta x/\Delta s \qquad (2)$$

where Δs is the biomass (g CDW/l) produced and Δx is the substrate uptaken (g/l) for the same time interval.

The biomass volumetric productivity (rX , g/l.d) was determined as follows:

$$rX = dx/dt \qquad (3)$$

where X corresponds to the biomass concentration (CDW, g/l), at a certain running time t (hours).

2.4. Results and Discussion

The selection of high cell densities strain remains an early step of improvement of biomass production. Shake flask assays and bioreactor validations were done in order to choose the strain that better metabolizes glycerol from biodiesel industry and reaches high cell concentrations.

2.4.1. Shake flask Tests

A preliminary shake flask screening was performed for cultivation of each *K. pastoris* strain, wherein glycerol byproduct was tested as the sole carbon source at different concentrations: runs E1 (43 g/l) and E2 (50g/l) that were compared with pure glycerol at 40 g/l (runs E0) (Figure 2.1 a) and b)).

In runs E0, using pure glycerol, strain DSM 70877 achieved slowly higher maximum wet cell weight (82g/l) than strain DSM 70382 (76 g/l) (Figure 2.1).

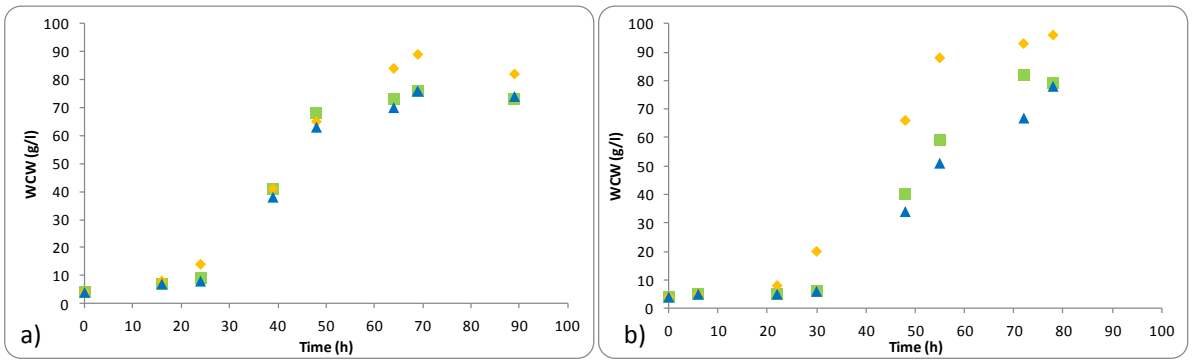

Figure 2.1- Wet cell weight (WCW) profile over time in the shake flask assays, for strains (a) *K. pastoris* DSM 70382 and (b) *K. pastoris* DSM 70877, using as carbon source (■, E0: 40 g/l pure glycerol), (♦, E1: 43 g/l glycerol byproduct) and (▲, E2: 50g/l glycerol byproduct).

Results obtained for cultivation of both strains in 43 g/l glycerol byproduct (runs E1), showed that *K. pastoris* DSM 70877 strain presented a faster growth, with higher maximum yeast cell growth, 95 g/l within 79 hours, compared to 89 g/l obtained within 69 hours for DSM 70382. Furthermore, the increased of glycerol byproduct concentration from 43 g/l to 50 g/l (runs E2) reveled a slower growth, with a lag-phase slowly higher, compared to growth on 43 g/l glycerol

byproduct as carbon source. Indeed, a decreased of wet cell weigh in DSM 70382 (76 g/l) and DSM 70877 (79 g/l) strains were observed, compared with results with glycerol byproduct at 43 g/l. These results may be related to the inhibition of bioconversion that may arise from considerable concentrations of substrates or products (high osmotic pressure in the fermentation medium) and/or the presence of toxic substances (metabolites and/or substrate impurities).

Based on these results, *K. pastoris* DSM 70877 was selected the most suitable *Komagataella* strain for cultivation at high cell densities, using the byproduct of biodiesel industry rich in glycerol as the sole carbon source on fed-batch culture.

2.4.2. Bioreactor cultivation

Considering the results obtained in the shake flask assays, namely, higher cell density, strain DSM 70877 was selected to perform fed-batch bioreactor experiments in a 2 l bioreactor, using glycerol byproduct (40g/l), as sole carbon source.

The run included an initial batch phase of 26 h (Figure 2.2), in which *K. pastoris* cells grew at a maximum specific growth rate of 0.12 h^{-1} and reached a biomass concentration of 22 g/l.

Table 2.1. - Comparison of μ_{max}, maximum specific growth rate and X_{max}, maximum biomass concentration reached at the end of fermentation in *A. niger*, *S. cerevisiae* and *K. pastoris*.

Microorganism	Carbons source	Cultivation Mode	$X_{máx.}$ g/l	$\mu_{máx.}$ (h^{-1})	Refs.
A. niger	Cane molasses	Batch	20	0.15	Feofilova et al., 2006; Ul-Haq et al., 2002
S. cerevisiae	Glucose	Fed-batch	120	0.20	Shang et al., 2006; Nguyen et al., 1998
K. pastoris	Glycerol byproduct	Fed-Batch	22*/104	0.17	This study

* Value of CDW(g/l) at the end of batch-phase.

As illustrated in Table 2.1, utilization of *K. pastoris* allows reaching very high biomass concentration during fermentation (much more difficult to attain with filamentous fungi, as *Aspergillus*, due to rheological constraints). Nevertheless, with a relatively lower growth rate (0.17 h^{-1}) (compared with other yeasts, such as *Saccharomyces* with μ_{max}=0.2 h^{-1} and X_{max} 120 g/l), using only glycerol as carbon source (much cheaper than glucose, for instance).

This corresponds to a growth yield of 0.55 g/g, similar to published results on pure glycerol (0.55 g/g) for recombinant *Pichia pastoris* strain and on glycerol byproduct (0.57 g/g) for *Pichia pastoris* E17 strain (Table 2.2). These results are consistent with observation by Çelik et al. (2008) (Table 2.2) where the cell yield on substrate using glycerol byproduct ($Y_{x/s}$ = 0.57 g/g) was higher than those attained with pure glycerol ($Y_{x/s}$ = 0.44 g/g).

After 26 h of cultivation, when glycerol was depleted, as indicated by a drop in the stirring rate (corresponding to a drop in oxygen consumption rate), the exponential feeding phase using glycerol byproduct and mineral media was initiated, resulting in an immediate increase in the oxygen consumption rate. Biomass concentration reached 104 g/l after 41 h of cultivation (Figure 2.2 and Table 2.1).

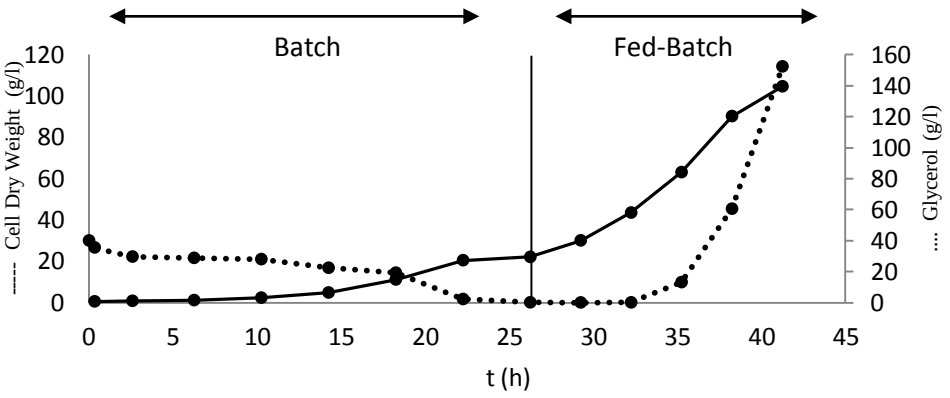

Figure 2.2- Evolution of biomass production (CDW) and glycerol concentration (g/l) during batch and fed-batch phases of the bioreactor cultivation of *K. pastoris*.

The growth yield increased slightly to 0.63 g/g during the fed batch phase (close to the value of 0.70 g/g reported by Jahic et al. (2002) for *Pichia pastoris* SMD1168 strain cultivated on four-stage process (including a batch with pure glycerol; the second stage was exponential fed-batch growth on glycerol; transiting to exponential fed-batch growth on methanol; followed a fourth stage with constant fed-batch growth on methanol used to control the oxygen demand at the

high cell density). Final cell concentration (104 g/l) was in the range of results obtained by other authors (75-120 g/l) with pure glycerol (Table 2.2). The overall biomass volumetric productivity was 60.9g/l.d.

Table 2.2- Comparison of biomass production and biomass yield in various strains of *P. pastoris*.

***P. pastoris* strain**	**Glycerol Source**	**Cultivation mode**	$X_{max.}$ **(g/l)**	$Y_{x/s}$ **(g/g)**	**Refs.**
E17	Glycerol byproduct	Fed-batch	10.5	0.57	Çelik et al., 2008
GS115	Pure	Fed-batch	75	n.d.	Chauan et al., 1999
Rec. *P. pastoris*	Pure	Fed-batch	120	0.55	Oliveira et al., 2005
SMD1168	Pure Glycerol/ Methanol	Batch/ Fed-batch	160	0.70	Jahic et al., 2002
DSM 70877	Glycerol byproduct	Fed-batch	104	0.55	This study

Rec.: Recombinant; n.d.: not determinated

These results confirmed that *Komagataella pastoris* DSM 70877 it is a promising strain: is able to reach very high biomass concentration (104 g/l) in the end of fed-batch culture on glycerol byproduct from the biodiesel industry, when compared with other strains (10.5 g/l) and showed a overall growth yield (0.55 g/g) closed to the observed by other authors (Table 2.2).

2.5 Conclusions

In the present study, two different *Komagataella pastoris* strains were cultivated in order to investigate the efficiency of using pure glycerol and glycerol byproduct from the biodiesel industry, in the growth biomass efficiency.

Shake flask assays results revealed that *Komagataella pastoris* DSM 70877 was able to efficiently grow on glycerol byproduct as the sole carbon source.

Strain DSM 70877 was selected to perform fed-batch bioreactor experiments due to the high growth biomass observed on shake flask assays.

Fed-batch cultivations with *K. pastoris* were performed using glycerol from biodiesel industry at 40 g/l as the sole carbon source. More than 100 g/l biomass was obtained in less than 48 h. The growth yield was 0.55 g/g during the batch phase and 0.63 g/g during the fed-batch phase. Results showed that glycerol from biodiesel industry can be valued by using them as sole carbon source to produce *K. pastoris* at high cell densities.

Chapter 3

Downstream Optimization and Polymer Characterization

Part of the results presented in this chapter were published in a peer reviewed paper:

Roca C., Chagas B., Farinha I., Freitas F., Mafra L., Aguiar F., Oliveira R., Reis M.A.M. (2012) Production of yeast chitin-glucan complex from biodiesel industry byproduct. Process Biochemistry, 47(11):1670-1675.

3.1 Summary

The yeast cell wall contains different polymers, including β-(1-3)-D-glucans, β-(1-6)-D-glucans, chitin and mannoproteins. Recent studies with *S. cerevisiae* suggest that the existence of covalent linkages between the different components of the wall, such as chitin and β-(1-3)-glucans (Kollar et al., 1997), as well as among glycoproteins, β-(1-6)-glucans and β-(1-3)-glucans, leads to the formation of different copolymers, such as, for example, glucomannans, galactomannans and chitin-glucan complex (CGC). The latter is a co-polymer of chitin and ß-glucans (Tarabukina et al., 2005).

K. pastoris showed significant amounts of CGC, in the range 18-26%, which makes feasible its extraction. Based on the work of Sugawara et al. (2004), several protocols were tested and optimized for the selective extraction of CGC by fractionation of *K. pastoris* biomass, followed by polymer purification. However, the fractions extracted had low degree of purity, thus requiring further extraction and purification steps. In order to do this, several assays were performed to increase extraction efficiency (extension of the reaction time, concentration of the alkali and temperature) and purification of CGC (different solvent washing procedures). It was found that the use of 5 M NaOH at 65 ºC, during 2 hours, associated to neutralization with HCl, increased CGC purity.

The obtained copolymer, CGC_{pure}, had a chitin:β-glucan molar ratio of 25:75. Residual contents of 1.5 wt.% mannose, 3.0 wt.% proteins and 0.9 wt.% inorganic salts were detected. Commercial CGC samples extracted from *A. niger* mycelium, kiOsmetine from Kitozyme, had higher inorganic salts (<5wt%) and protein (<8 wt%) contents (www.kitozyme.com).
CGC_{pure} had a chitin:β-glucan molar ratio similar to the values reported for kiOsmetine, 30:70. CGC_{pure} was characterized by Differential Scanning Calorimetry (DCS) and solid-state Nuclear Magnetic Resonance (NMR) spectroscopy and compared with commercial biopolymers, namely, crab shell chitin and chitosan, algal β-glucan (laminarin) and commercial fungal CGC (kiOsmetine). However, it had distinct thermal properties, namely a single narrower

endothermic peak, indicating the presence of minor proteins and the absence of salts, comparatively to the commercial CGC biopolymer.

3.2 Introduction

The yeast cell wall is a complex of different macromolecules, mainly composed by polysaccharides that correspond for over 50% of the cell dry weight. There are three main groups of polysaccharides that form the yeast cell wall: polymers of mannose covalently linked to peptides (mannoproteins, 30-50%), polymers of glucose (1,6-β-glucans: 5-10% and 1,3-β-glucans: 30-45%) and lower amounts of polymers of *N*-acetylglucosamine (chitin, 1.5-6%) that is usually present in the form of a chitin-glucan complex (Figure 3.1) (Orlean, 1997 and Klis et al., 2005).

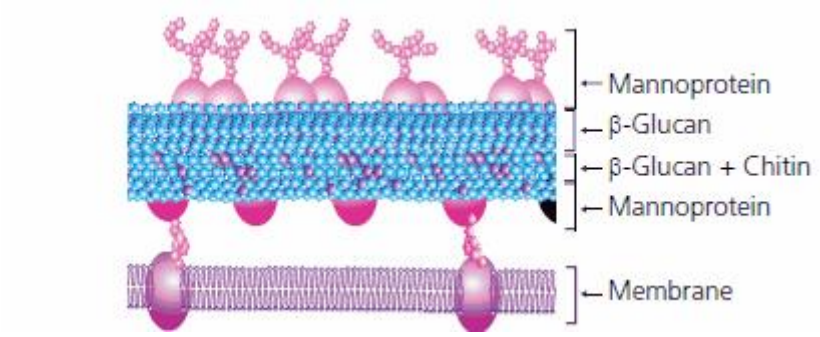

Figure 3.1- Yeast cell wall composition (Sigma-Aldrich).

Extraction of the polysaccharides from higher organisms (plants, algae and animals) it is the most frequent, however, presents many constraints due their seasonal character, with highly variable quality and quantity, making their production processes particularly irreproducible. Therefore, polysaccharides with distinct composition, such as CGC can be extracted from the yeast cell wall cultivated under controlled conditions, representing a valuable source and makes the process more reliable, sustainable and safe (Synowiecki and Al-Khateeb, 2003; Sugawara et al., 2004).

CGC extraction from yeast cell walls consists of two stages:

1. Yeast cell lysis: CGC is localized in the cell wall, thus it is necessary to lyse cells and separate the insoluble cell wall fraction from the cytoplasm, lipids, DNA, proteins and debris;
2. Cell wall fractionation of the cell lysed components by their selective solubilization to obtain different polysaccharides, such as CGC and polysaccharides containing different sugar monomers, such as glucose, mannose and/or galactose (Klis et al., 2005; JohnSton, 1965; Otero et al., 1996; Synowiecki and Al-Khateeb, 2003; Sugawara et al., 2004).

At present, CGC is isolated from fungi and yeasts through physicochemical and/or enzymatic methods (Feofilova et al., 2002) with the use of inorganic reagents (Kanarskaya, 2000) in combination with synthetic detergents (Kotlyar, 2001), and methods based on the use of organic solvents and detergents (Feofilova et al., 1995; Ivshina et al., 2007; Sugawara et al., 2004).

An interesting alternative to chemical methods is the enzymatic method. CGC can be enzymatically extracted from the *Aspergillus niger* mycelium, a byproduct of the production of pharmaceutical and food-grade citric acid (Versali et al., 2003). The extraction and purification process is environmentally friendly – it uses no animal- or synthetic-derived reactants and has minimal environmental impact. However, residual protein often remains high and reaction times are significantly increased compared to chemical methods. Enzyme costs are further more prohibitive, limiting enzymatic methods in industrial applications (Percot et al., 2003).

In recent years, increasing attention has been paid to CGC isolated from the cell wall of yeast and fungi (Versali et al., 2003; Ivshin et al., 2007; Ivshina et al. 2009). In respect to the high incidence of medical applications (Su et al., 1997; Gautier et al., 2008), various methods were used to purify CGC from the yeast cell wall during the last decades: Nguyen et al. (1998) used

an alkaline hydrolysis, Ha et al. (2002) used alkaline–acid hydrolysis, and Magnelli et al. (2002) used the sequential treatment of yeast and fungi cell walls with specific hydrolytic enzymes, followed by dialysis.

Studies on the chemistry and structure of yeast walls have focused mainly on *Saccharomyces cerevisiae* and *Candida albicans*. For those species, as well as for most other yeasts that have been examined, the cell wall consists of about 85±9.0% polysaccharide, namely water soluble mannans, alkali soluble glucans, alkali insoluble glucans and small amounts of chitin and 10±1.5% protein, often covalently linked to the mannan, which is more correctly described as mannoprotein.

In the present study, a method to optimize the purification of CGC from *K. pastoris* cell wall was developed. In this context, several tests were performed to remove contaminants (mannose-containing polymers, protein and ashes) from CGC, including washing with water, PBS (phosphate-buffered saline solution), ethanol and/or HCl neutralization. Nguyen et al. (1998) showed that PBS can be efficiently used to remove proteins during cell wall preparation for fractionation because it is a good solvent for most proteins.

A minor amount of lipids (5-15% of dry weight) is found in cell wall preparations. Organic solvent extraction is the method most commonly used for lipid extraction, because of its economic and technical advantages, such as its high selectivity and solubility toward lipids, and the low cost of solvents. The use of ethanol at cold temperature had been tested for evaluate the effects on extraction of lipids from yeasts, since induces the rupture of most polar interactions between lipids and proteins (Gerde et al., 2012). However, this method presents the disadvantage of protein precipitation along with insoluble polymer fractions. Other methods for lipid extraction include chemical solvent extraction. Yu et al. (2014) suggested that acid digestion, such as with HCl, may be the most simple and effective method for lipid recovery (47.3%) from the tested species. Furthermore, the use of HCl for neutralization of alkaline-

insoluble fraction, for purification of CGC has been applied by several authors (Ivshin et al., 2007; Du et al., 2012).

Washing steps improve the removal of the solubilized cell wall components in the extract, namely, proteins (by washing with water and/or PBS solution and/or HCl solution), lipids (by washing with ethanol and/or HCl solution), and salts (by washing with water and/or HCl solution). The appropriate choice of the type of solvent system(s), the number of washing steps performed and the sequence by which they are performed is used to control the content of proteins, lipids and ashes in the extracted CGC.

Thus, *K. pastoris* biomass fractionation using different methods was performed and their impact on CGC composition was evaluated. To the best of our knowledge, this study was the first systematic description of the wall composition of *Komagataella pastoris* yeast.

3.3 Materials and methods

3.3.1 Fractionation of Komagataella pastoris cell wall

Firstly, a procedure to fractionate the biomass into different polysaccharide fractions was tested (Fig 2). The procedure was based on the methods described by Sugawara et al. (2004), with some modifications.

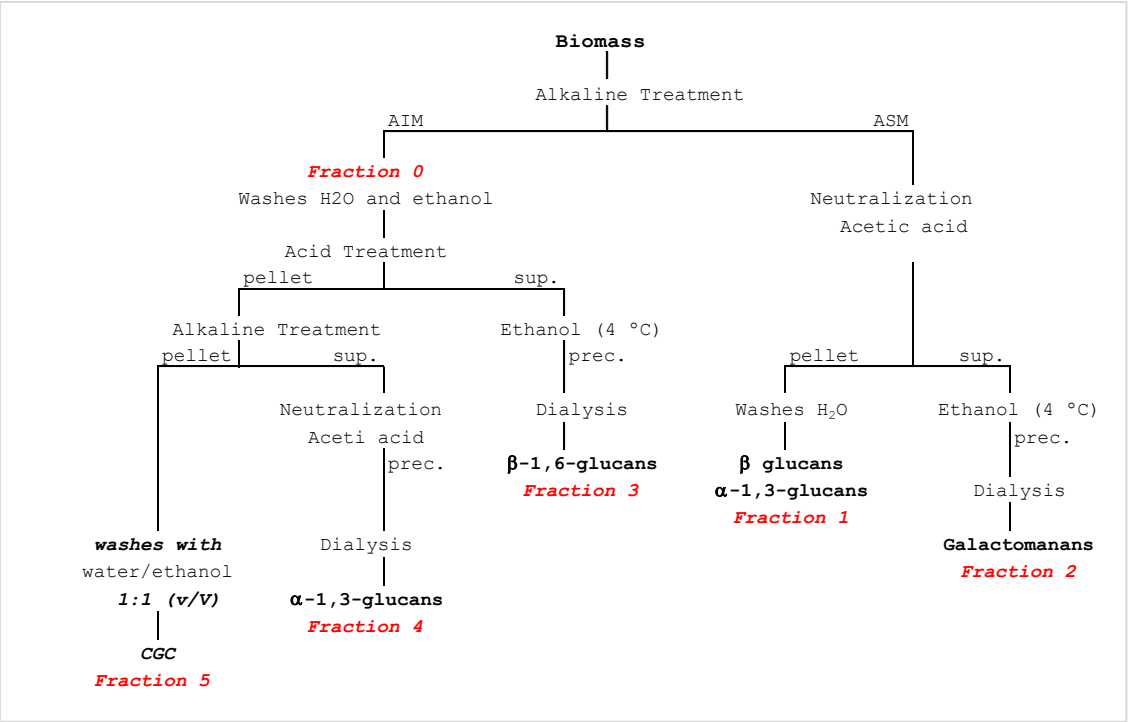

Figure 3.2- Diagram of the process of extraction of cell wall constituents of yeast.

ASM (alkaline soluble material); AIM (alkaline insoluble material).

The biomass was subjected to an alkaline treatment with 1 M NaOH at 65 ºC, for 2 h, for cell disruption. The suspension had a biomass content of 10% (w/v) on a dry basis.

The mixture was centrifuged at 10 000×*g* for 10 minutes at 4ºC, to separate the resulting alkaline insoluble material (AIM) from the alkaline soluble material (ASM). After that, selective extractions were done in order to obtain different alkali insoluble polysaccharides, such as β-(1-6)-glucans, α-(1-3)-glucans and CGC, as well as alkali soluble polysaccharides, such as β-glucans, α-(1-3)-glucans, mannans and galactans.

Extraction of alkaline soluble polysaccharides

The ASM was neutralized (pH 6.5) by addition of 1M acetic acid. This led to the precipitation of water insoluble polymers (α-1,3-glucans) that were separated from the soluble ones (galactomannans) by centrifugation (10 000×*g*, for 10 minutes at 4ºC). The precipitated α-1,3-glucans were repeatedly washed with distilled water in order to remove proteins and lipids. Finally, the recovered α-1,3-glucans were lyophilized for 24 hours (fraction 1; Figure 3.2).

The water soluble polysaccharides (galactomannans - fraction 2 in Figure 3.2) present in the ASM were precipitated by the addition of ethanol (1:1 v/v), with constant stirring. Given that precipitation is favored at low temperatures, this was carried at 4 °C. The precipitated polymers were resuspended in deionised water 10% (w/v). Residual alkali, as well inorganic salts and other low molecular weight impurities, were removed from impure fraction 2 by dialysis. Dialysis was carried out through a semi-permeable membrane (Snakeskin dialysis tubing; Cut-Off 10 KDa) against deionized water, with 10 ppm sodium azide, to avoid microbial contamination, at 4 ºC, until the conductivity was ≤ 20 μS/cm. The dialyzed solution was centrifuged at 10 000 rpm for 10 minutes and then lyophilized for 24 hours.

Extraction of alkaline insoluble polysaccharides

The AIM (fraction 0) was subjected to an acid treatment to dissolve acid soluble polysaccharides (β-1,6-glucans) (Figure 3.2).

To this fraction 0, 30 ml of a 1 N HCl solution per gram of sample were added, and the mixture was kept at 30 °C, for 1 hour. After this time, the mixture was centrifuged (10 000×*g* for 10

minutes at 4ºC). The acid-soluble polymers present in the supernatant were precipitated by the addition of ethanol, at 4 ºC. The sample was re-suspended in deionized water and dialyzed as described above for fraction 2. The resulting polymers, branched β-1,6-glucans, were lyophilized for 24 hours (fraction 3, Figure 3.2).

To guaranty the separation of the alkaline soluble polymers and proteins, the pellet obtained by the acid treatment was submitted to a second hot alkaline treatment with 30 ml/g_{sample} 1 N NaOH solution (at 75 °C for 1 hour). In order to avoid deacetylation of chitin, the temperature was kept below 90 °C and the reaction took less than 3 hours. The mixture was centrifuged (10 000×*g* for 10 minutes at 4ºC). The pellet containing CGC (fraction 5 in Figure 3.2) was washed with 30 ml of water and ethanol (1:1 v/v) and, finally, it was lyophilized for 24 hours. The polysaccharides present in the supernatant (α-1,3-glucans, fraction 4) were precipitated with ethanol, after neutralization (pH 6.5) by addition of 1M acetic acid, dialyzed at the same conditions as for fraction 2 and lyophilized for 24 hours.

3.3.2 Purification of CGC

Following the polysaccharides fractionation described in section 3.3.1., different procedures were tested to obtain *K. pastoris* CGC (fraction 5) with a higher purity degree.

*3.3.2.*1 Optimization of Alkaline Treatment

In an attempt to simplify and make CGC extraction method faster, sustainable and economic, several assays were performed using a one-step alkaline treatment with different NaOH concentrations, temperatures and reaction times, as well as various solvent washings.

Purification of CGC (effect of solvent washing)

CGC was extracted from the yeast biomass (10-30 mg) by alkaline treatment with NaOH 1 M (7 ml) at 65 ºC, for 2 h (method I, Table 3.1). Then, the AIM was recovered by centrifugation (10 000 ×*g*, for 15 min), washed twice with 200 ml of deionized water, twice with the same volume of PBS (20.45 g/l NaCl; 0.46 g/l KCl; 10.14 g/l Na_2HPO_4. $7H_2O$; 0.54 g/l KH_2PO_4, pH 7.2) and one wash with ethanol for the removal of lipids. A final wash with deionized water was performed in order to remove ethanol and residual salts. The resulting polymer was lyophilized for 24 hours.

In method II (Table 3.2), CGC was extracted from the yeast biomass (10-30 mg) by alkaline treatment with NaOH 1M (7 ml) at 65 ºC, for 2 h. The AIM was resuspended in deionised water and the pH of the suspension was adjusted to 7.0 by the addition of HCl 6 M. Then, it was repeatedly washed with deionised water (200 ml). The pH and conductivity of the suspension were monitored during the washing procedure, which ended when the pH and conductivity were 7.0 and ≤20µS/cm, respectively. The resulting CGC was freeze-dried (48h).

Table 3.1- Different CGC alkaline extraction assays, with different solvent washings.

Method	**Alkaline treatment**			**Solvent washing**
	NaOH (M)	**T (ºC)**	**Time (h)**	
I	1	65	2	2×water→ 2x PBS→ ethanol → water
II	1	65	2	Neutralization(HCl 6M)→ water*
III	1	65	0	Neutralization(HCl 6M)→ water*
IV	1	65	5	Neutralization(HCl 6M)→ water*
V	1	80	2	Neutralization(HCl 6M)→ water*
VI	0	65	2	Neutralization(HCl 6M)→ water*
VII	5	65	2	Neutralization(HCl 6M)→ water*

*repeated washing with deionized water, until constant pH and conductivity values were reached.

Effect of reaction time

The biomass was suspended in hot 1 M NaOH solution 10% (w/v) (65 ºC). The mixture was kept at 65 ºC for 5 h with stirring (method IV, Table 3.1). For comparison, in method III, the biomass was suspended in hot NaOH 1 M and subjected to the same solvent washing as method II.

Effect of temperature

The test the effect of temperature on CGC extraction efficiency, it was performed at 80 ºC (method V, Table 3.1) and compared with method II that was performed at 65 ºC. Temperature had to be lower than 90 °C to prevent occur deacetylation of chitin.

Effect of NaOH concentration

To test the effect of alkali concentration, the biomass was suspended in 5 M NaOH. The mixture was heated at 65 ºC for 2 h with stirring (method VII, Table 3.1). For comparison, in method V the biomass was suspended in deionized water and subjected to the same procedure of method VII.

3.3.3. Polymer characterization

Chemical characterization of K. pastoris cell wall Fractions

All the fractions obtained as described in section 3.3.1. (Figure 3.1) were analyzed for their sugar composition, as well as the presence of proteins, ash content and moisture. For the sugar

compositional analysis, the fractions were subjected to two distinct acid hydrolysis procedures: trifluoroacetic acid (TFA) was used to hydrolyse the glucan moiety of the polymer, while a stronger acid (HCl) was necessary for the quantification of the chitin fraction. For the TFA hydrolysis, dried samples (~5 mg) were resuspended in deionised water (5 ml) and 0.1 ml TFA 99% were added. The hydrolysis was performed at 120 ºC, for 2 h. For the HCl hydrolysis, the samples (~5 mg) were resuspended in HCl 12 N (7.5 ml). The hydrolysis was performed at 120 ºC, for 5 h. Both hydrolysates were used for the quantification of the constituent monosaccharides by HPAEC-PAD chromatography (ICS-3000 system DIONEX), using a CarboPac PA10 column (Dionex), equipped with an amperometric detector, (electrochemical detector- pulsed integrated amperometric detection with working electric gold and AgCl reference cell).

The analysis was performed with sodium hydroxide (NaOH 18 mM) as eluent, at a flow rate of 0.8 ml/min and carried out at 30 ºC. Glucose (Sigma), mannose (Sigma) and glucosamine (Sigma) were used as standards at concentrations between 0.2 and 0.005 g/l, being subjected to the same hydrolysis procedures as the polymer samples.

A specific enzyme glucan analysis kit (K-YBG) from Megazyme) [www.megazyme.com] was used to quantify α-glucans, and total glucose. The amount of β-glucan was calculated from the difference of the two. Commercial reference CGC polymers isolated from the mycelium of the fungus *Aspergillus niger* (KyosmetineCG.30 and KyomedineCTU, with glucans contents in the range 46-65 mol% and 21 mol%, respectively), from Kitozyme were used as controls. A control yeast β-glucan (from kit - K-YBG) was also used.

Protein and inorganic salt contents

Dried polymer samples were hydrolyzed with 2 M NaOH (7 mg:1 ml) at 120 °C for 15 min. The

supernatant obtained by centrifugation (10 000 x *g* 10 minutes) was used for the protein assay, according to the modified Lowry method (Stoscheck, 1990). A 1-ml aliquot of alkaline copper sulfate reagent was added to 1 ml of the supernatant (diluted when necessary) and allowed to stand for 10 min at room temperature. A 3-ml aliquot of diluted Folin–Ciocalteu reagent was added, and incubated for 30 min at room temperature. Absorbance was read at 750 nm. Bovine serum albumin (BSA, Sigma) at concentrations between 0 and 5,0 g/l, was used as standard to generate the calibration curves.

The inorganic salts content of the samples was evaluated by subjecting them to pyrolysis at a temperature of 550 ºC for 48 h.

Elemental analysis

CGC extracted from *K. pastoris* cell wall and kiOsmetine (the fungal CGC commercialized by Kitozyme), crab-shell chitin (Fluka), crab-shell chitosan (Sigma) and laminarin (Sigma) were analyzed for their carbon, hydrogen, and nitrogen content, using the elemental Analyzer Thermo Finnigan-CE Instruments (Italy), model Flash EA 1112 CHNS.

Thermal properties

The Differential Scanning Calorimetry (DSC) analyses were conducted with a Setaram Calorimeter (model DSC 131, France) under a protective nitrogen gas atmosphere. Accurately weighed dried material was placed in an aluminum cup and hermetically sealed. The measurements were carried out from 25 to 450 ºC under nitrogen at a scanning rate of 10

ºC/min. Samples of commercial polymers, namely, crab shell chitin and chitosan, laminarin and kiOsmetine, were also analyzed under the same conditions.

Solid-state Nuclear Magnetic Resonance

^{13}C spectra were recorded on a 9.4 T WB (400 MHz, ^{1}H Larmor frequency) Bruker Avance III spectrometer. A 4 mm double-resonance MAS probe was employed at 400.1 (^{1}H) and 100.6 MHz (^{13}C) Larmor frequencies. Samples were spun in ZrO2 rotors using a spinning rate of 12 kHz. ^{13}C CPMAS NMR experiments were employed a ramp cross-polarization (CP) step (varying from 100% to 50% in amplitude using 100 points); contact time (CT): 1.5 ms; 1H 90◦ excitation pulse: 3.75 µs; ^{1}H and ^{13}C RF field strength for CP was set to 87 kHz and 68 kHz, respectively; NS: 1k; recycle delay: 5 s. ^{13}C Chemical shifts are quoted in parts per million (ppm) and calibrated with respect to, the external reference, glycine (C O, 176.03 ppm).

3.4 Results and Discussion

3.4.1 *Fractionation of Komagataella pastoris cell wall*

With the ultimate goal of obtaining CGC, *K. pastoris* biomass was first subjected to a fractionation procedure to evaluate its content in different cell-wall polysaccharides.

Optimization of biomass deproteination process is a key role to obtain biopolymers, namely CGC, with high purity. Several procedures were tested to improve the removal of solubilized cell wall components in the biomass extract. The appropriate choice of the type and concentration of solvent system(s), the duration and temperature of reactions, the number of

washing steps performed and the sequence by which they are performed is important to control the biopolymer´s content in contaminants, such as proteins, lipids and ashes.

A fractionation of cell wall polysaccharides presented in section 3.3.1. involved an initial hot alkali extraction (NaOH 1 M, 2 h at 65 ºC), followed by acid neutralization of the ASM and acid treatment (HCl 1 M, 1 h at 30 ºC) of the AIM.

The yields of the various fractions are shown in Figure 3.3-A) and the composition of each fraction is shown on Figure 3.3-B).

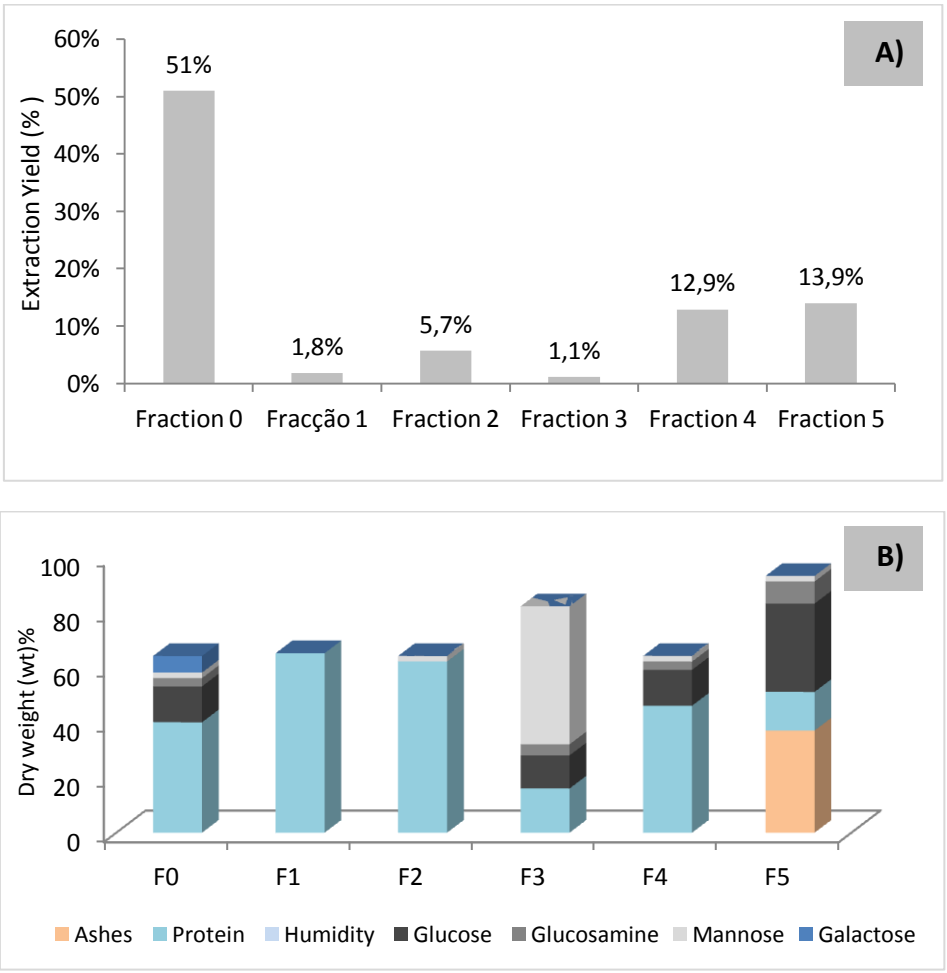

Figure 3.3- Yield extraction of different fractions in *K. pastoris* (3-A); Composition of different fractions of polymers (3-B).

An extraction yield of 27.9% of alkali insoluble polysaccharides was obtained from *K. pastoris* cell wall (fractions 3, 4 and 5), while considerably lower yield of alkali soluble polymers (7.5%) was obtained (fractions 1 and 2). CGC content in *K. pastoris* cells (fraction 5) was 13.9 wt.% (Figure 3.3-A).

Fractions 1 and 2, obtained from ASM, are mostly composed of protein (65 and 62 wt.%), respectively, with minor contents of mannose (2 wt.%) in fraction 2. Remaining components of fractions 1 and 2, probably correspond to lipids, DNA and cellular debris, which were solubilized by the alkaline treatment and carried over to the ASM.

The AIM obtained after the alkaline treatment of the biomass (fraction 0) was mainly composed of proteins (40 wt.%), with a sugar content of 24 wt.%, including 13 wt.% glucose, 2 wt.% mannose, 6 wt.% galactose and 3 wt% glucosamine (Figure 3.3-B). Three main fractions were obtained from fraction 0 (AIM): (1) fraction 3 that was rich in mannose (50 wt.%) and proteins (16 wt.%), but with some glucose (12 wt.%), corresponding to alkali soluble β-glucans, probably linked to glucosamine (4 wt.%); (2) fraction 4, was mainly composed of proteins (46 wt.%), with a sugar content of 18 wt.%, including 13 wt.% glucose, corresponding at α-1,3-glucans, 2 wt.% mannose and 3 wt% glucosamine (Figure 3.3-B); (3) Fraction 5 had glucose and glucosamine contents of 32wt.% and 8 wt.%, respectively, as well as a total protein content of 14 wt.%, a mannose content of 4 wt.%, and an inorganic salts content of 37 wt.%. This fraction 5 corresponded to the CGC extracted from *K. pastoris* biomass. It had a chitin:β-glucan molar ratio of 20:80. The high values of contaminants (inorganic salts) detected in this fraction might have been due to the treatment with solvents during the extraction procedures.
Commercial CGC (kiOsmetine from Kitozyme) had a much lower ash contents (<5 wt%), as well as reduced protein content (<8 wt%) (www.kitozyme.com).

The biomass content in glucans was 14 % (as determined by the K-YBG enzyme kit, Megazyme), while fraction 5 had a content of about 34 %, corresponding to glucose covalently linked to chitin (Table 3.2). Results were confirmed by its proximity to the results obtained by HPLC analysis (32 %).

Table 3.2- Summary of the results obtained by quantification of glucan per gram of polymer using the enzymatic kit from Megazyme (K-YBG) and by HPLC analysis.

		Kit K-YBG	**HPLC Analysis**	**Certificate of Analysis (mol%)**
Control	KiOsmetineCG.30	40%	52	46 - 65
	KiOmedineCTU	23%	18	21
	Control Yeast β-Glucan	68%	60	73
Fraction 5 (CGC)	*K. pastoris*	34%	32%	-

Though results regarding extraction of CGC from *K. pastoris* yeast seem very interesting, high values of contaminants (ashes and proteins) proved that extraction protocols were not optimized. Thus, the next step aimed at the development of extraction and purification protocols in order to obtain CGC with a higher purity degree.

3.4.2 Purification of CGC

K. pastoris biomass was exposed to hot alkaline treatment with different alkali concentration, namely 1 and 5M NaOH, different reaction times (0, 2 and 5 h), temperatures (65 and 80 ºC) and different solvent washing (water/PBS/ethanol/water washing or neutralization with HCl/water washing) to assess the effect of these factors on protein solubilization and,

consequently, on CGC purity. The aim of this part of the work was to get the highest purity of CGC and the lowest concentration of contaminants, such as mannose, proteins and inorganic salts, in an alkaline-insoluble material using as low as possible concentration of alkali solution. Indeed, using the method described in section 3.3 the obtained CGC contained high amounts of contaminants (14 wt.% of protein, 4 wt.% of mannose and 37 wt.%. of inorganic salts) which need to be removed.

Effect of solvent washing system on CGC purification efficiency

In order to evaluate the effects of the use of different solvent washing systems PBS/ethanol/water system washing (method I, Table 3.3) or neutralization with HCl followed washing of CGC with water (method II, Table 3.3), two assays were performed. Data showed evidences on the use of these different solvent washing systems, after deproteination with 1 M NaOH, at 65 ºC, during 2 h. Method II improved the removal of the solubilized cell wall components of biomass, namely, mannose containing polymers and proteins, as well as salts. In fact, mannose containing polymers were decreased from 28 wt%, using method I, to 6.3 wt%, using method II. Similarly, the polymer's protein and ash contents were reduced from 9.5 and 15wt% (method I) to 7.2 and 1.5wt% (method II). These results could be justified by the use of ethanol, in method I, which led to the precipitation of mannoproteins, who were dragged along with CGC. In turn, neutralization with HCl 6 M, followed by washings with water, improved the removal of the solubilized cell wall components in the extract, such as proteins, mannans and inorganic salts.

Table 3.3- Effect of temperature, NaOH concentration, reaction time and solvent washing on the efficiency of the alkaline treatment for CGC extraction from *K. pastoris* biomass.

Method	Alkaline treatment			Extraction yield (wt%)	Chitin:Glucan Molar Ratio (%mol)	Mannose (wt%)	Protein (wt%)	Inorganic salts (wt%)
	NaOH (M)	T (ºC)	Time (h)					
I	1	65	2	26	16:84	28.0	9.5	15
II	1	65	2	21	8:92	6.3	7.2	1.5
III	1	65	0	64	19:81	48.1	14.0	n.a.
IV	1	65	5	16	25:75	2.3	3.0	1.8
V	1	80	2	18	8:82	4.6	3.0	n.a.
VI	0	65	2	79	32:68	44.7	18.0	n.a
VII	5	65	2	14	25:75	1.5	3.0	0.9

n.a.: not analyzed.

Effect of reaction time on CGC purification efficiency

CGC extraction efficiency based on reaction time (methods II, III and IV; Table 3.3) was analyzed. NaOH 1 M was used and the reactions were performed for 0, 2 and 5 h. Indeed, by increasing the reaction time from 2 h (method II) to 5 h (method IV), CGC extraction yields decrease from 21 wt% to 16 wt%, which was due to the decrease in the amount of contaminants (proteins, mannose and inorganic salts). CGC resulting from methods II and IV presented higher purity when compared to fraction 5 described in section 3.4.1. The protein content in the CGC decreased with the reaction time from 14 wt% (at 0 h) to 3wt% (at 5 h). Furthermore, the chitin:β-glucan molar ratio decreased from 8:92 to 25:75 mol% with the increase of time reaction from 2 to 5 h. This decrease can be related with the fact that increasing the reaction time favored solublization of alkaline-soluble polymers or, on the other hand, may suggest partial degradation of glucans linked to chitin.

Effect of temperature on CGC purification efficiency

After determination of the reaction time conditions for CGC recovery, the extraction process based on optimal temperature extraction was analyzed. Data (methods II and V from table 3.3) showed that optimal temperature conditions for higher amounts of CGC (21 wt%) was 65 °C. At 80 ªC the amount of CGC was lower (18 wt%) but had slightly less amounts of contaminants. Both methods showed the same chitin:β-glucan molar ratio (8:92 mol%). Given these results, the temperature of 65 ºC was selected since it allowed saving energy.

Effect of NaOH concentration on CGC purification efficiency

Experimental data (Table 3.3) show that conditions to obtain a better CGC extraction efficiency were: 5M NaOH concentration at 65 ºC and reaction time of 2 h. Furthermore, remaining proteins and inorganic salts also decreased from 7.2 to 3.0 wt %, and from 1.8 to 0.96 wt %, respectively, using 1 or 5 M NaOH solutions (methods II and VII, Table 3.3). These results suggested that 5 M NaOH, at 65 ºC, during 2 hours, followed by neutralization with HCl and water washing, improve the removal of alkaline-soluble fraction (method VII, Table 3.3). Under these conditions, the extraction procedure was improved when compared with fraction 5 obtained in section 3.4.1., particularly in relation to contaminants. Fraction 5 had a chitin:β-glucan molar ratio of 20:80, but high contaminants contents: mannans (2 wt.%), proteins (14 wt.%) and ashes (37 wt.%). With Method VII, a CGC with a molar chitin:β-glucan ratio of 25:75 mol% with residual contents of 1.5 wt.% mannans, 3 wt.% proteins and 0.9 wt.% inorganic salts was obtained. kiOsmetine, the commercial fungal CGC had higher ash contents (<5wt%), as well as protein content (<8 wt%) (www.kitozyme.com).

3.4.3 CGC chemical characterization

The biopolymer extracted from *K. pastoris* biomass with method I, CGC_{PBS}, (Table 3.3) had glucose and glucosamine contents of 35 and 7 wt%, respectively, as determined by the compositional analysis performed with the TFA and HCl hydrolysates. These values correspond to a chitin:β-glucan molar ratio of 16:84, which is lower than the values reported for kiOsmetine (between 30:70 and 50:50) (Gautier et al., 2008). The lower ratio of chitin:β-glucan found for *K. pastoris* CGC_{PBS} may be related to the extraction procedure that resulted in an impure polymer that probably contained alkali soluble glucans that were not efficiently removed during the extraction procedure. Mannose containing polysaccharides were also not completely removed, as shown by the high mannose content of CGC (28 wt%). Moreover, it also had a total protein content of 9.5 wt%, as well as an inorganic salts content of 15.0 wt%. Indeed, kiOsmetine had a much lower mannose content (1.7 wt%), as well as reduced protein (<8 wt%) and ash (<5 wt%) contents (www.kitozyme.com).

The copolymer obtained with method VII, CGC_{pure}, presented a chitin:β-glucan molar ratio of 25:75 (mol%), which is close to the values reported for kiOsmetine, with chitin:β-glucan molar ratios of 30:70. The higher ratio obtained for *K. pastoris* CGC_{pure} may be related to the extraction and purification procedures that resulted in an pure polymer, as shown by the total protein content of 3.0 wt% as well as an inorganic salts content of 0.9 wt%, which are lower than the commercial CGC ((protein content <8 wt%) and ash content <5 wt%) (www.kitozyme.com).

Elemental analysis of *K. pastoris* CGC_{PBS} and of several commercial biopolymers is presented in Table 3.4. Crab-shell chitin and chitosan from (Sigma-Aldrich) were used as comparative reference of polymers composed of monomers of glucosamine with different degrees of acetylation. Laminarin (Fluka) was used as soluble β-glucan reference and KiOsmetine is a commercial fungal CGC.

The highest nitrogen content was found in crab-shell chitin and chitosan, while no nitrogen was detected in laminarin sample (as expected for a polymer composed only of glucose units). Comparing to kiOsmetine, *K. pastoris* CGC_{PBS} had lower nitrogen content. In such samples, both chitin and protein contributed to the presence of nitrogen. *K. pastoris* CGC_{PBS} had a protein content of 9.5%, which, in theory, corresponds to about 1.5% of nitrogen (Ivshina et al., 2009). Hence, the nitrogen content attributable to chitin is rather low, but consistent with the low content of chitin in the biopolymer (7%).

Table 3.4- Elemental analysis of produced CGC_{PBS} compared with laminarin, crab shell chitosan, crab shell chitin and commercial kiOsmetine.

Biopolymer	Elemental analysis (%)		
	C	H	N
CGC	49.50	8.27	1.73
KiOsmetine	41.17	7.18	2.70
Laminarin	38.02	6.94	0.00
Crab shell chitin	44.97	7.16	6.71
Crab shell chitosan	40.51	7.54	7.14

Furthermore, the extraction yield (13.4 %) obtained for CGC_{pure} is similar to the values reported for other fungi, such as *Aspergillus niger* (12-25 wt%) (Table 3.5), while the chitin content was much higher than that reported for *Saccharomyces cerevisiae* (3 wt%).

Table 3.5- CGC content in *A. niger*, *S. cerevisiae* and *K. pastoris* (%CGC, g CGC/g dry cell biomass; % chitin, g chitin/g CGC).

Microorganism	Carbon source	% CGC	% Chitin	Refs.
A. niger	Cane molasses	12-25	38-41	Feofilova et al., 2006; Ul-Haq et al., 2002
S. cerevisiae	Glucose	25	3	Shang et al., 2006; Nguyen et al., 1998
P. pastoris	Glycerol byproduct	13.4	25	This study

3.4.4 Thermal Properties

The thermal properties of the CGC samples isolated from *K. pastoris* (CGC_{PBS} and CGC_{pure}) were analyzed using DSC from 25 to 450 ºC. Closely related commercial biopolymers were included in the thermal analysis as a comparison: laminarin (algal glucan), crab shell chitin and chitosan, and the fungal CGC kiOsmetine. As shown in Figure 3.4, each biopolymer presented a broad endothermic peak around 50–100 ºC, which can be attributed to the evaporation of the water bound to the polysaccharides (Kittur et al., 2002; Prashanth et al., 2002).

CGC_{PBS} and Kiosmetine presented a strong endothermic peak, suggesting that these molecules presented a high water holding capacity, the strongest affinity being for the CGC_{PBS} produced. On the contrary, chitin, laminarin and CGC_{pure} presented a much weaker peak. DSC analysis allows measuring the purity of CGC: generally pure crystals melting show broad endothermic peaks whereas purer larger crystals show narrow endothermic peaks.

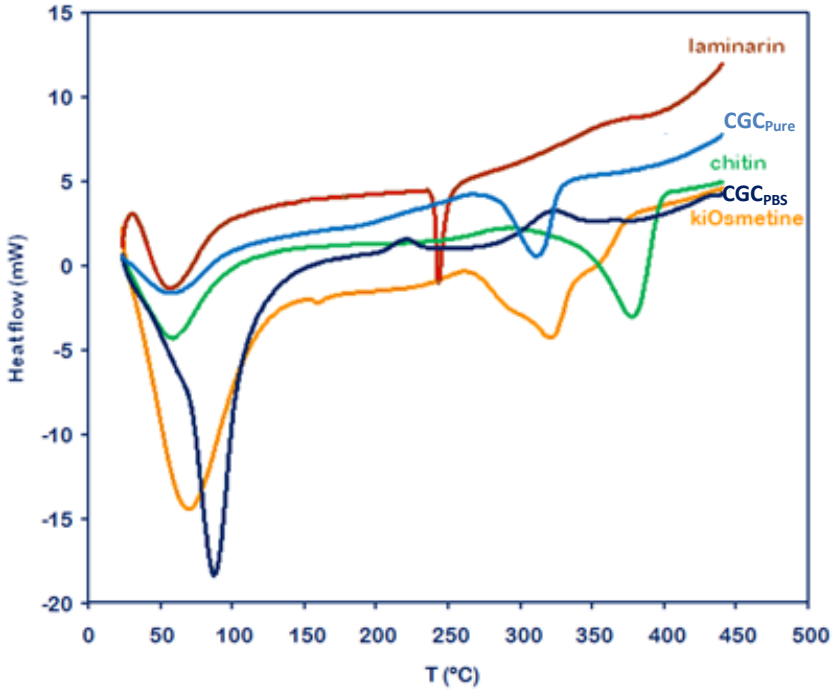

Figure 3.4- DSC scans of produced CGC_{PBS}, CGC_{pure}, laminarin, crab shell chitosan, crab shell chitin and commercial kiOsmetine.

K. pastoris CGC_{PBS} showed two decomposition exothermic peaks at 205.18 and 288.38 ºC. The very low enthalpy (lower peak) indicates that phase transformation is difficult, for CGC_{PBS} would suggest a biomaterial with very low crystallinity, whereas sample of chitosan (with high narrow exothermic peak) was highly crystalline (Yen and Mau, 2007).

The presence of two exothermic decomposition peaks in CGC_{PBS} is difficult to interpret but could suggest the presence of two polymers, probably a mixture of chitin:β-glucan and mannans and/or glucomannans, usually associated to yeast cell walls (and detected during the compositional analysis).

Laminarin and chitin showed clear endothermic peaks at 237 and 378 ºC, respectively, whereas kiOsmetine showed a broader endothermic peak at 324.16 ºC, suggesting the presence of impurities (salts and proteins) in the commercial CGC biopolymer.

CGC_{pure} showed a single narrower endothermic peak, indicating the presence of minor proteins and the absence of salts, comparatively to the commercial CGC biopolymer and to the CGC_{PBS}. These results showed that method VII is more effective to obtain a CGC free of proteins, mannans and/or glucomannans than obtained in the method I.

Additionally, thermogravimetric measurements of CGC_{pure} and CGC_{PBS} presented an endothermic decomposition peak at 315 ºC and 312 ºC, respectively, assigned to the corresponded degradation temperature (T_{deg}) of the polymers (Farinha et al, 2015). Kaya, Erdogan, Mol & Baran (2015) reported to crustacean chitin a T_{deg} of 381-385 ºC, while a lower T_{deg} have been reported for yeast β-glucans, as *Saccharomyces cerevisiae* β-glucan, where the onset temperature of the decomposition is 267 ºC (Novak et al., 2012).

3.4.5 Solid-state NMR

To understanding the molecular-scale structure and dynamics of macromolecules contents on CGC polymer, the extracted polymer CGC_{PBS} and CGC_{pure} were characterized by solid-state Nuclear Magnetic Resonance (NMR) spectroscopy and compared with commercial biopolymers, namely, crab shell chitin, algal β-glucan (laminarin) and kiOsmetine.

The former spectrum of contains ^{13}C resonances typical of a chitin:β–glucan mixture (see Figure 3.5 for ^{13}C resonance labeling). For example, the ^{13}C peaks appearing at the chemical shifts (δ) of 103.3, 74.4 and 61.9 ppm correspond, respectively, to the anomeric C1, C2, 3 and C6 chemical environments of the β-glucan monomers.

The ^{13}C resonances from the chitin monomers are also clearly observed in the same spectrum at δ ~172.9, 55.3 and 22.9 ppm, which are assigned to the carbonyl group (C7′ from acetyl groups), the quaternary carbon (C2′) involved in a C-N bond, and the methyl group (C8′, from acetyl groups) (Figure 3.5a). It is worth mentioning that a comparison with our produced

chitin:β–glucan biopolymer, extracted with PBS (Figure 3.5a) or neutralized with 1M HCl (Figure 3.5b) with other biopolymers containing (i) pure β-glucan (laminarin, Figure 3.5d); (ii) pure chitin (Figure 3.5e) and (iii) a commercial β-glucan-chitin (Figure 3.5c) strongly supports the ^{13}C resonance identification made, thus, confirming that purified CGC biopolymer is essentially a superposition of the commercial laminarin (Figure 3.5d) and the crab shell chitin (Figure 3.5e) ^{13}C spectra. Moreover, the ^{13}C spectrum of purified CGC shows approximately the same resonance positions as the ^{13}C spectra of the commercial biopolymer obtained from Kitozyme (Figures 3.5b and c), which indicates that both chemical structures are very similar. Some unassigned peaks due to not identified polymer components are indicated with asterisks in Figure 5b (e.g., the ^{13}C peaks resonating at δ ~30.4, 32.9 and 128.7 ppm), which are also observed in other commercial biopolymers (Figures 3.5c, d and e). Such unidentified components may also arise from residual amounts of proteins and/or lipids, as observed by others (Cervera et al., 2004; Spevacek and Brus, 2008).

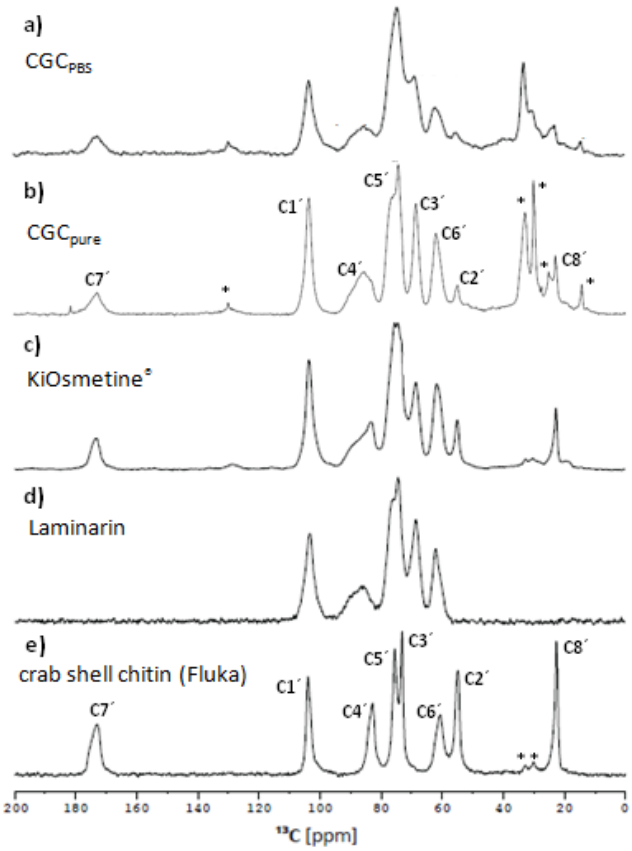

Figure 3.5- ^{13}C CPMAS spectra of (a) the biopolymer extracted from I biomass compared with (b–d) other commercial biopolymers. Asterisks denote impurities. Selected carbon resonance assignments are depicted in Fig. 3.1; numbers inside square brackets depict carbon resonances of the β-glucan polysaccharide involved in β-1,3 glycosidic bonds. * A carbon resonances involved in β-1,6 glycosidic bonds.

Results show that the CGC_{pure} contains less chitin than β- glucan monomers with respect to the commercial biopolymer from Kitozyme. This may be observed by the weaker intensity of C8´ and C2´ chitin ^{13}C resonances with respect to the glucan ^{13}C resonances existent in our extracted biopolymer (Figures 3.5a) and b), which is consistent with the results of chemical characterization, previously discussed.

3.5 Conclusions

In the present chapter, CGC was extracted from *K. pastoris* biomass cultivated on crude glycerol from the biodiesel industry.

A method using sequential fractionation of *K. pastoris* biomass was developed to evaluate its content in different cell-wall polysaccharides. The biomass had CGC content of 13.9 wt.% (fraction 5), which was considered interesting. However, the presence of high values of contaminants (14 wt% of proteins and 37 wt% of inorganic salts) in the polymer, showed that extraction protocols were not optimized to obtain a CGC with market application.

Different methods were tested for CGC extraction and purification, aiming at obtaining a pure CGC copolymer, with low contents of contaminants.

Yields of the AIM (after the treatment of the *K. pastoris* biomass with 1 – 5 M NaOH solutions at 65 – 80 ºC, during 2-5 h) were in the range 14 - 21% on a dry cell weight basis, according to conditions of the procedure. The most promising results were achieved by using 5 M NaOH solution at 65 ºC during 2 h.

Further improvements on purification steps were necessary to obtain the biopolymer with higher purity (by further reducing the amount of contaminants as detected in solid-state NMR or suggested by DSC of CGC_{PBS}).

Simple hot alkaline extraction method followed by solvent washing and neutralization with HCl led to a biopolymer composed of a chitin:β-glucan molar ratio of 25:75 with a content of 1.5 wt.% mannose, 3 wt.% proteins and 0.9 wt.% inorganic salts. Commercial CGC, kiOsmetine from Kitozyme, had higher ash contents (<5wt%), as well as reduced protein content (<8 wt%) (www.kitozyme.com).

A preliminary physical characterization of the CGC_{pure}, by solid-state NMR spectroscopy and DSC, revealed a molecule close to *A. niger* CGC with, however, distinct thermal properties. CGC_{pure} showed a single narrower endothermic peak, indicating the presence of minor proteins and the absence of salts, comparatively to the commercial CGC biopolymer.

To the best of our knowledge, this is the first time that glycerol was used to produce CGC from *K. pastoris* biomass. This yeast is widely used to produce heterologous proteins but the generated biomass is seldom valorized. Valorization of yeast biomass from industrial process could be a way to produce CGC at low cost, with relatively high chitin content, using only glycerol byproduct as carbon source. *K. pastoris* represents a novel source of polysaccharides with industrial and medical applications.

Chapter 4

Effect of pH and temperature on chitin-glucan complex production

The results presented in this chapter were published in a peer reviewed paper:

Chagas, B., Farinha, I., Galinha, C., Freitas, F., Reis, M. (2014) Chitin-glucan complex Production by *Komagataella* (*Pichia*) *pastoris*: impact of cultivation pH and temperature on polymer content and composition. New Biotechnol, 31(5):468-74.

4.1 Summary

In this work, the yeast *Komagataella (Pichia) pastoris* was grown on glycerol as the sole carbon source in batch cultivation experiments to evaluate the effect of pH (3.5–6.5) and temperature (20–40 ºC) on CGC production and polymer composition. The CGC content in the biomass and the volumetric productivity (r_p) were not significantly affected within the tested pH and temperature ranges. Nevertheless, both parameters could be maximized (CGC ≥ 14 wt% and r_p ≥ 3.0 gCGC/l·day) for temperatures within 27-34 ºC and pH above 6.0 or below 4.0. In contrast, the effect of pH and temperature on the polymer's chitin:β-glucan molar ratio was more pronounced. The highest chitin:β-glucan molar ratio (> 14:86) was obtained for the mid-range pH (4.5-5.8) and temperatures (26–33 ºC), while a drastic reduction of chitin to ≤ 6 mol% was observed outside those ranges. Therefore, a compromise between maximal CGC production and the synthesis of polymers enriched in chitin must be achieved, depending on the final application of this product.

4.2 Introduction

Chitin accounts for 1 - 2% of dry cell weight of yeasts and up to 40% in filamentous fungi (Klis et al., 2006), but it is known to be subject to considerable fluctuations as a response to different cell wall stress conditions, including, for example, high temperature (Kamada et al., 1995; Russo et al., 1993), hypotonic shock (Davenport et al., 1995; Tomita et al., 1996), and exposure to heavy metals (Pestov et al., 1999) or cell wall binding agents (Garcia et al., 2004; Martin et al., 2000). Some studies have been made regarding the effect of cultivation pH and temperature on chitin content in certain yeast and fungi strains (Aguilar-Uscanga et al., 2003; Hsieh et al., 2007; Smits et al., 1998).

Temperature

Temperature is one of the most important physical parameters which influence yeast growth. Microbial cells require specific temperature conditions, since they have an optimum temperature for their metabolic activity and they do not have the ability to regulate their internal temperature. Temperatures over the optimum will accelerate cell death and at the opposite direction will slow down metabolic reaction kinetics (Donati and Sand, 2007). Therefore it is preferred to keep the medium temperature at the optimum value throughout the bioprocess. Otherwise, not only the reaction rates, but also metabolic regulations, nutritional requirements, biomass composition, product formation and yield coefficients will be affected by temperature; however, the optimum temperature for growth and product formation and productivities may be different. On the other hand, when temperature is increased above the optimum temperature, the maintenance requirements of cells will also increase, and becomes a stress factor for cells. Fermentation processes with *K. pastoris* are usually run at an optimum temperature for growth of 30 °C (Wegner, 1983).

pH

Other physical growth requirement for yeast cells is pH, the hydrogen ion concentration. It plays important role in the activity of enzymes, transport mechanisms and other extracellular and intracellular events as in the case of temperature therefore it influences the growth rate. Microbial cells have a remarkable ability to maintain the intracellular pH at a constant level,

even with large variations in the pH of the extracellular medium, but only at the expense of an increase in the maintenance energy demands, since Gibbs free energy has to be used for maintaining the proton gradient across the cell membrane. (Nielsen and Villadsen, 1994).

Most yeast species grow very well between pH values of 4.5 and 6.5 (Walker, 1998). *K. pastoris*, particularly, is capable of growing across a relatively broad pH range from 3.0 to 7.0 which actually does not affect the growth significantly (Macauley-Patrick et al., 2005).

Nevertheless, there is no universal trend, as each strain apparently shows different cell wall composition adaptations in response to those environmental factors. For example, the chitin content in *S. cerevisiae* cells has been reported not to be significantly affected by pH in the range 3.0-6.0, but it rose as the growth temperature increased from 22 to 37 ºC (Aguilar-Uscanga et al., 2003). In contrast, Hsieh et al. (2007) reported that the fungi *Rhizopus oligosporus* BCRC 31996, *Monascus pilosus* BCRC31527 and *Aspergillus* sp. BCRC31742 changed the cells' chitin content as a response to cultivation pH (between 3.0 and 7.0). Smits et al. (1998) also found that chitin content in *Trichoderma reesei* QM9414 cells increased as the temperature was raised from 20 to 32 ºC, but it declined for higher temperatures (> 34 ºC).

Komagataella pastoris is commonly cultivated with pH and temperature control at 5.0 and 30 ºC, respectively (Roca et al., 2012; Soyaslan and Çalik, 2012; Çelik et al., 2008), but the strain has been reported to be able to grow at a wide pH (3.0-7.0) (Çalik et al., 2010; Cos et al., 2006) and temperatures (15-25 ºC) ranges (Çelik et al., 2008; Gasser et al., 2007; Wu et al., 2012; Li et al., 2001). At temperatures above 32 ºC protein expression stops, hence they are not commonly used for most *K. pastoris* processes (Cos et al., 2006). Although the effect of nutritional and environmental factors on growth and protein expression by *K. pastoris* has been extensively studied (Soyaslan and Çalik, 2012; Çalik et al., 2010; Cos et al., 2006; Gasser et al., 2007; Chiruvolu et al., 1998; Files et al., 2001; Inan et al., 1999; Shi et al., 2003), their impact on this yeast cell wall composition, namely, on CGC content and chitin:β-glucan ratio, was not assessed.

The aim of this work was to study the impact of cultivation temperature (20-40 ºC) and pH (3.5-6.5) on *K. pastoris* cell wall content in CGC and on the polymer's chitin:β-glucan ratio. Response surface methodology (RSM) was used to evaluate the interactive effect of the two parameters and determine the most appropriate ranges to improve CGC productivity, as well as to obtain a polymer enriched in chitin.

4.3 Materials and Methods

4.3.1. Yeast strain and medium

Komagataella pastoris DSM 70877 was cultivated in standard basal salts medium (BSM) (Pichia Fermentation Process Guidelines, Invitrogen), as described in chapter 2. BSM was supplemented with glycerol (~86% w/v) to give a concentration of 50.0±5.0 g/l.

4.3.2. Bioreactor operation

Inocula for bioreactor experiments were prepared by incubating the culture in BSM medium, containing glycerol (~40 g/l) in shake flasks for 3 days at 30 ºC, in an incubator shaker (200 rpm). 10% vol/vol inocula were used to inoculate the 5 l bioreactor (BioStat B-plus, Sartorius), which was operated with controlled temperature and pH in the range of 20-40 ºC and 3.5-6.5, respectively, for the different runs, according to the experimental design (Table 1). pH was controlled by the automatic addition of 25% (v/v) ammonium hydroxide solution that served also as the nitrogen source. The dissolved oxygen (DO) concentration was controlled at 50% by the automatic variation of the stirring rate (between 300 and 2000 rpm) and supplementation of the air stream with pure oxygen. All experiments were performed in a batch mode. Samples (20-25 ml) were periodically withdrawn from the bioreactor during the experiments for determination of the cell dry weight, glycerol concentration, CGC content in the biomass and polymer composition.

4.3.3. Analytical techniques

Cell separation from fermentation broth samples were collected as described in chapter 2. Four replicas were used for determination of the CDW.

The cell-free supernatant was stored at -20ºC for the determination of glycerol concentration. Glycerol concentration in the cell-free supernatant was determined by liquid chromatography (HPLC) as described in chapter 2.

For extraction of CGC from the yeast biomass, freeze dried biomass samples (10-30 mg) were treated with NaOH 5 M (7 ml) at 65 ºC, for 2 h, for solubilisation of cell wall components. The alkali-insoluble material obtained by centrifugation of the mixture (10 000 × *g*, for 15 min) was

re-suspended in deionised water and neutralised with HCl 6 M. After centrifugation (10 000 × *g*, for 15 min), the polymer was washed twice with deionised water to remove alkali soluble components and, finally, it was freeze dried for the gravimetric quantification of the polymer content in the biomass. Three replicas were used for this analysis.

The determination of CGC sugar composition was performed by acid hydrolysis, using trifluoroacetic acid (TFA) and hydrochloric acid (HCl) to hydrolyse the glucan and the chitin fractions of the polymer, respectively, as described in chapter 2. Both hydrolysates were used for the quantification of the constituent monosaccharides by HPLC, using a CarboPac PA10 column (Dionex), equipped with an amperometric detector. The analysis was performed at 30ºC, with sodium hydroxide (NaOH 4 mM) as eluent, at a flow rate of 0.9 mL/min. Glucose (Sigma) was used as standard for the quantification of the glucan fraction of the CGC polymer. Glucosamine (Sigma) was used as standard for quantification of the chitin content of the polymer (under the hydrolysis conditions used, namely, concentrated HCl, chitin was depolymerized and de-N-acetylated, resulting in the formation of the monomer glucosamine (Einbu and Vårum, 2008; Artamonova and Sharnina, 2013).

4.3.4. Kinetic parameters

The maximum specific cell growth rate (μ_{max}, h^{-1}) and the CGC volumetric productivity (r_p, gCGC/l·day) were determined using the equations described in chapter 2.

2.5. Experimental design

Response surface methodology (RSM) (Lundstedt et al., 1998), using a central composite rotatable design (CCRD) with two factors and three replicas of the central point, was used to evaluate the optimal cultivation conditions for CGC production by *K. pastoris*. This procedure was used to understand the overall effect and interaction of experimental variables (X_i): pH and temperature (T, ºC), and the observed responses (Y_i): specific growth rate (μ, h^{-1}); CGC biomass content (CGC, wt%); CGC volumetric productivity (r_p, gCGC/l·day), and the polymer's chitin:β-glucan molar ratio.

This design was composed of eleven experiments (Table 4.1) that were performed randomly: four factorial design points at levels ±1; four experimental of axial level $\alpha = \pm 1.414$; and a central point with three replicas. Using CCRD, the independent variables were expressed as a second order model:

$$Y_p = b_0 + b_1X_1 + b_2X_2 + b_{11}X_1^2 + b_{22}X_2^2 + b_{12}X_1X_2 \quad (4)$$

where Y_p corresponds to the predicted responses, X_1 and X_2 are the coded values of the independent variables; b_0, b_i, b_j, b_{ij} (i, j = 1,2) are the regression coefficients, where b_0 is the interception, b_1 and b_2 the linear terms, b_{11} and b_{22} the quadratic terms and b_{12} the interaction term.

This model was fitted to experimental data through statistical analysis (analysis of variance – ANOVA, and multiple linear regression – MLR). The statistical analysis was done using the software Statistica, version 6.0 (StatSoft Inc., Tulsan, UK). The fitted model [Eq. (4)] was evaluated for each response variable based upon the multiple correlation coefficients (R^2), regression parameter significance (p-value) and tested lack of fit. To be considered a good predictive tool, the model should satisfy the following criteria: a good correlation value (R^2 > 0.7), which is acceptable for biological samples, according to Lundstedt et al. (1998), with statistical meaning (p-value < 0.05, for a 95% confidence level) and with no lack of fit (p-value > 0.05, for 95% confidence level), i.e. the model error was in the same range as the pure error. The factors and their interaction were also evaluated by p-value at 95% confidence level. The effect of temperature and pH on the response was given by statistics and the surface plots analysis.

4.4 Results and Discussion

4.4.1. Influence of temperature and pH: response analysis

Table 4.1 summarizes the results obtained in the eleven experiments preformed in this study. Under the central point conditions (pH = 5.0 and 30 ºC; experiments 9, 10 and 11), high specific cell growth rates were obtained (0.17-0.18 h^{-1}) for the batch cultivation of *K. pastoris* on glycerol. As expected, the results show that growth was not significantly affected by altering the cultivation pH (experiments 3 and 4), although a slight reduction of the maximum specific cell growth rate (μ_{max} = 0.12 h^{-1}) was noticed for the highest pH value tested (pH = 6.5; experiment 4). In contrast, lowering the cultivation temperature (20–22.9 ºC; experiments 1, 5 and 6) seemed to have a severe effect upon cell growth, which was considerably slower (μ_{max} = 0.04-0.08 h^{-1}). On the other hand, temperatures above the central point conditions (experiments 7 and 8) had a lower impact on the specific cell growth rate (μ_{max} = 0.12-0.14 h^{-1}), except at 40 ºC (experiment 2), where no growth was observed (Table 4.1).

The CGC content in the biomass was not significantly affected by most of the conditions tested (Table 4.1). Indeed, for most of the experiments of this study, the CGC content (11-14 wt%) was within the range of values obtained under the central point conditions (12-14 wt%). Nevertheless, there was an increase of the CGC content to 17-20 wt% in experiments 3, 5 and 7, which were conducted at lower pH values (3.5–3.9).

Table 4.1- Central composite rotatable design (CCRD) with two independent variables X_1 (temperature, T) and X_2 (pH), and the observed responses studied: Y_1, maximum specific growth rate (μ_{max}); Y_2, CGC content in the biomass (CGC); Y_3, CGC volumetric productivity (r_p), and Y_4, polymer's chitin:β-glucan molar ratio.

Experiment	T (ºC)	pH	CDW (g/l)	μ_{max} (h^{-1})	CGC (wt%)	r_p (gCGC/l·day)	chitin:β-glucan molar ratio
	X_1	X_2		Y_1	Y_2	Y_3	Y_4
1	20.0	5.0	19	0.04	11	0.60	5:95
2	40.0	5.0	15	0.00	…	0.00	…
3	30.0	3.5	48	0.17	20	4.89	4:96
4	30.0	6.5	58	0.12	16	3.76	11:89
5	22.9	3.9	54	0.05	17	1.54	7:93
6	22.9	6.1	54	0.08	12	3.92	5:95
7	37.1	3.9	29	0.14	20	3.69	0:100
8	37.1	6.1	40	0.12	14	4.02	5:95
9	30.0	5.0	34	0.17	12	3.44	15:85
10	30.0	5.0	45	0.18	14	4.03	15:85
11	30.0	5.0	22	0.17	13	2.53	17:83

The overall volumetric productivity (r_p) of the process (3.69-4.02 gCGC/l·day) was within the range of the values obtained under the standard conditions (2.53-4.03 gCGC/l·day), except for experiments 1 and 5, for which r_p was lower (Table 4.1). Nevertheless, r_p was improved in experiment 3 (pH = 3.5 and 30 ºC), wherein it attained its maximum value (4.89 gCGC/l·day) in this study.

Although the CGC content in the biomass and the volumetric productivity did not seem to be dramatically affected for most of the conditions tested in this study, the polymer's composition was significantly altered (Table 4.1). In all experiments, the chitin:β-glucan molar ratio in the CGC was lower (≤ 11:89) than in the polymer obtained under the central point conditions (15:85–17:83).

4.4.2. Response surface methodology

The effect of temperature or pH on the different responses evaluated by the one-to-one factor did not allow the identification of the interactions between the two variables, which was only possible by the use of response surface methodology (RSM). Using statistical analysis, the working pH and temperature ranges to achieve the highest CGC productivity were defined. Moreover, the conditions for production of CGC with a targeted chitin:β-glucan molar ratio were also established.

Table 4.2- Analysis of variance (ANOVA) of the central composite design: model and lack of fit significance levels (*p*-values) and correlation values (R^2) for the responses studied (maximum specific growth rate (μ_{max}, h^{-1}); CGC content in the biomass (CGC, wt%); CGC volumetric productivity (r_p, gCGC/l· day), and the polymer's chitin:β-glucan molar ratio).

Effect	Model	Lack of fit	R^2
	p-value	*p*-value	
μ_{max} (Y_1)	0.036	0.018	0.856
CGC (Y_2)	0.220	0.031	0.675
r_p (Y_3)	0.156	0.239	0.725
Chitin:β-glucan (Y_4)	0.001	0.320	0.967

RSM (ANOVA and MLR) analysis for the several responses is summarized in Table 4.2. The second order model developed had a good correlation factor ($R^2 > 0.70$), acceptable for biological samples (Lundstedt et al., 1998), for all responses, except for CGC content in the biomass (Y_2) ($R^2 = 0.675$).

Nevertheless, the parity plots (Figure 4.1) show that the correlation between the experimental and predicted values have slopes close to 1, which indicates that, despite some dispersion of values, the models obtained capture the behavior of the experimental data. In fact, the experimental data obtained in triplicate for the central point reveals some error associated to the experiments, supporting the observation of data dispersion in the parity plots. Moreover, there were no obvious outlier data since all values are within the confidence intervals (defined as 2-times the standard deviation of the output experimental data).

The ANOVA *p*-values showed that the second order model had significance ($p < 0.05$) for the specific growth rate (Y_1) and the chitin:β-glucan molar ratio (Y_4) responses, but not for the CGC content in the biomass (Y_2) nor the volumetric productivity (Y_3) (Table 4.2). For the specific growth rate (Y_1) and the CGC content in the biomass (Y_2) responses, the model prediction error was above the error of the replicas, as evidenced by the lack of fit ($p < 0.05$). However, in those specific cases, the lack of fit could be explained by the pure error (calculated with the replicates of the central point), which was close to zero, thus giving an artificial sense of a model with lack of fit.

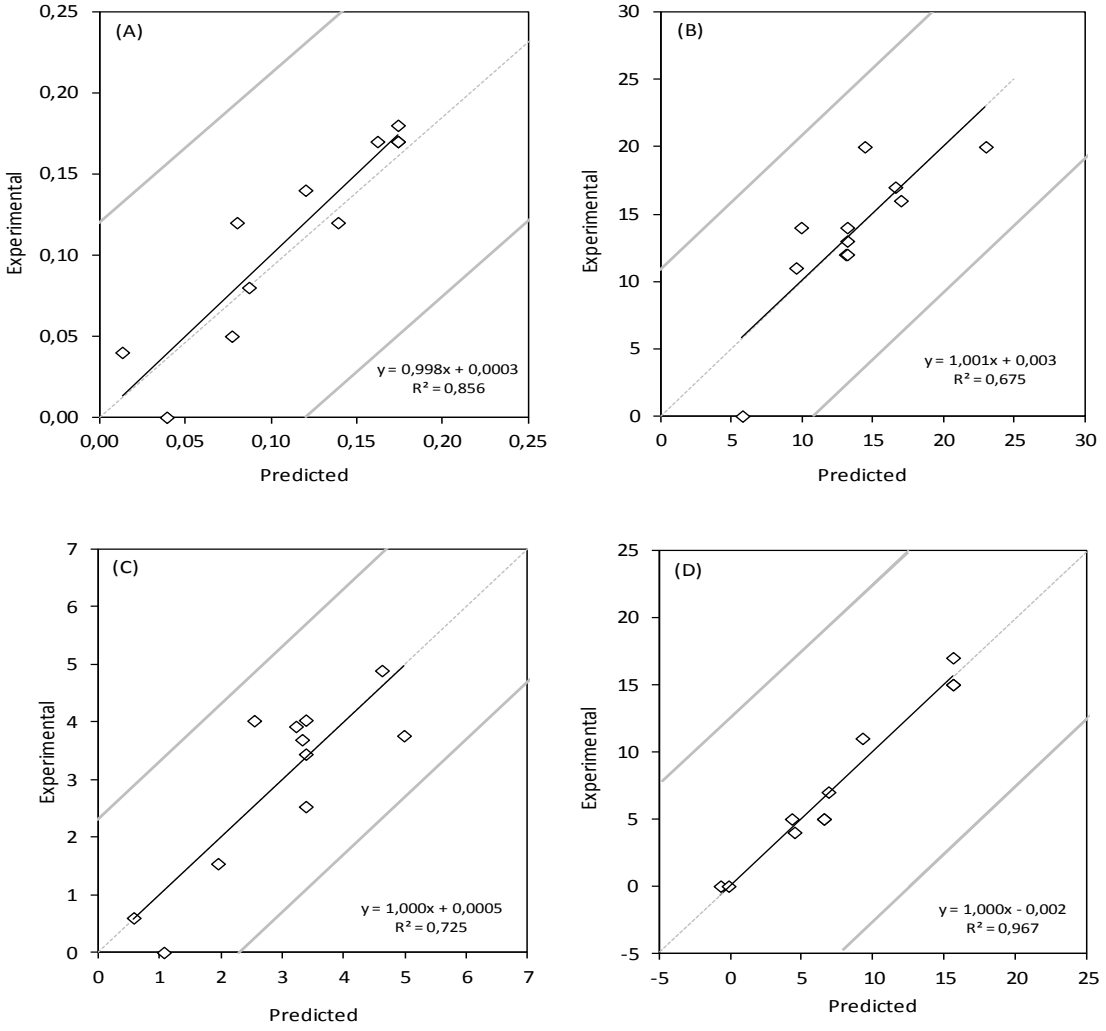

Figure 4.1- Parity plots for observed and predicted values for the responses studied (maximum specific growth rate (A), CGC content in the biomass (B), CGC volumetric productivity (C) and polymer's chitin:β-glucan molar ratio (D)).

Effect of pH and temperature on cell growth

The linear, quadratic and interaction effects of temperature and pH on the maximum specific cell growth rate (μ_{max}), evaluated by MLR, are given in Table 4.3 and represented in Figure 4.2-A. It can be observed that the interaction between pH and temperature was negligible for this response (Y_1) ($p > 0.05$) (Table 4.3).

Table 4.3 - Multiple linear regression (MLR) analysis of the polynomial models: regression coefficients (normalized values) and *p*-values for linear, quadratic and interaction effects of temperature (T) and pH for the responses studied (maximum specific growth rate (μ_{max}, h^{-1}); CGC content in the biomass (CGC, wt%); CGC volumetric productivity (r_p, gCGC/l· day), and the polymer's chitin:β-glucan molar ratio).

Effect		Linear		Quadratic		Interaction
	Constant	T (X_1)	pH (X_2)	T×T (X_1^2)	pH×pH (X_2^2)	T×pH (X_1X_2)
$_{\mu max}$ (Y_1)	0.174	0.013	-0.011	-0.148	-0.023	-0.027
p-value	0.000	0.475	0.532	0.003	0.455	0.485
CGC (Y_2)	13.195	-1.894	-3.000	-5.506	6.792	-0.536
p-value	0.003	0.430	0.242	0.201	0.134	0.914
r_p (Y_3)	3.380	0.246	0.179	-2.557	1.420	-1.098
p-value	0.004	0.693	0.778	0.051	0.220	0.421
chitin:β-glucan (Y_4)	15.604	-2.500	2.382	-13.800	-8.736	3.750
p-value	0.001	0.023	0.029	0.000	0.001	0.071

Moreover, μ_{max} was not influenced by pH, being affected mostly by the quadratic term of temperature (T×T) ($p < 0.05$) (Table 4.3). This is represented by the parabola format of the surface plot (Figure 4.2-A), in which growth was predicted to be very slow ($\mu_{max} < 0.02\ h^{-1}$) for extremes of temperature (T < 22ºC and T > 38 ºC). The model predicted that, under the conditions tested, *K. pastoris* achieved higher cell growth rates ($\mu_{max} \geq 0.16\ h^{-1}$) within temperature and pH ranges of 28–34 ºC and 3.0-5.8, respectively.

These results are in accordance with previous studies that investigated the effect of temperature and/or pH on *K. pastoris* growth on glycerol. Indeed, this strain's specific cell growth rate has been reported to be not significantly affected by pH within the range 3.5-5.5 (Çalik et al., 2010; Cos et al., 2006). Narrower temperature ranges (28-34 ºC) have also been reported for optimal growth of *K. pastoris* (Çelik et al., 2008; Cos et al., 2006; Gasser et al., 2007). However, none of the previous works evaluated the effect of temperature and pH on CGC content.

Effect of pH and temperature on CGC production

According to the model, both the temperature and pH had negligible impact on the CGC content in the biomass (Figure 4.2-B) and the volumetric productivity (Figure 4.2-C) ($p > 0.05$ for all terms) (Table 4.3). However, the model estimated that both responses could be maximized (CGC $\geq$ 14wt% and $r_p \geq 3.0$ gCGC/l·day) for a rather narrow temperature range (27-34 ºC) (Figures 4.2-B and 4.2-C). On the other hand, optimal CGC production and volumetric productivity was predicted to be achieved by controlling pH above 6.0 or below 4.0. These results, suggesting a narrow range for temperature and opposite limiting conditions for pH, may thus explain the low significance of models for the CGC content in the biomass (Y_2) and for the volumetric productivity (Y_3) (Table 4.2), as well as the apparent low impact of T and pH on that parameters (Table 4.3).

Effect of pH and temperature on CGC chitin:β-glucan molar ratio

The model showed that both the temperature and the pH influenced the chitin:β-glucan molar ratio of the polymer (Figure 4.2-D), with the linear and quadratic terms (T×T and pH×pH) having a great impact ($p < 0.05$) (Table 4.3). The interaction terms had negligible impact ($p > 0.05$). Hence, it can be predicted that production of polymer enriched in chitin (chitin:β-glucan molar ratio > 14:86) is favored by temperature and pH within the ranges 26 - 33ºC and pH 4.5 - 5.8, respectively (Figure 4.2-D). Outside those ranges, especially for T < 21ºC and T > 38ºC, the chitin:β-glucan molar ratio was considerably reduced ($\leq$ 6:94).

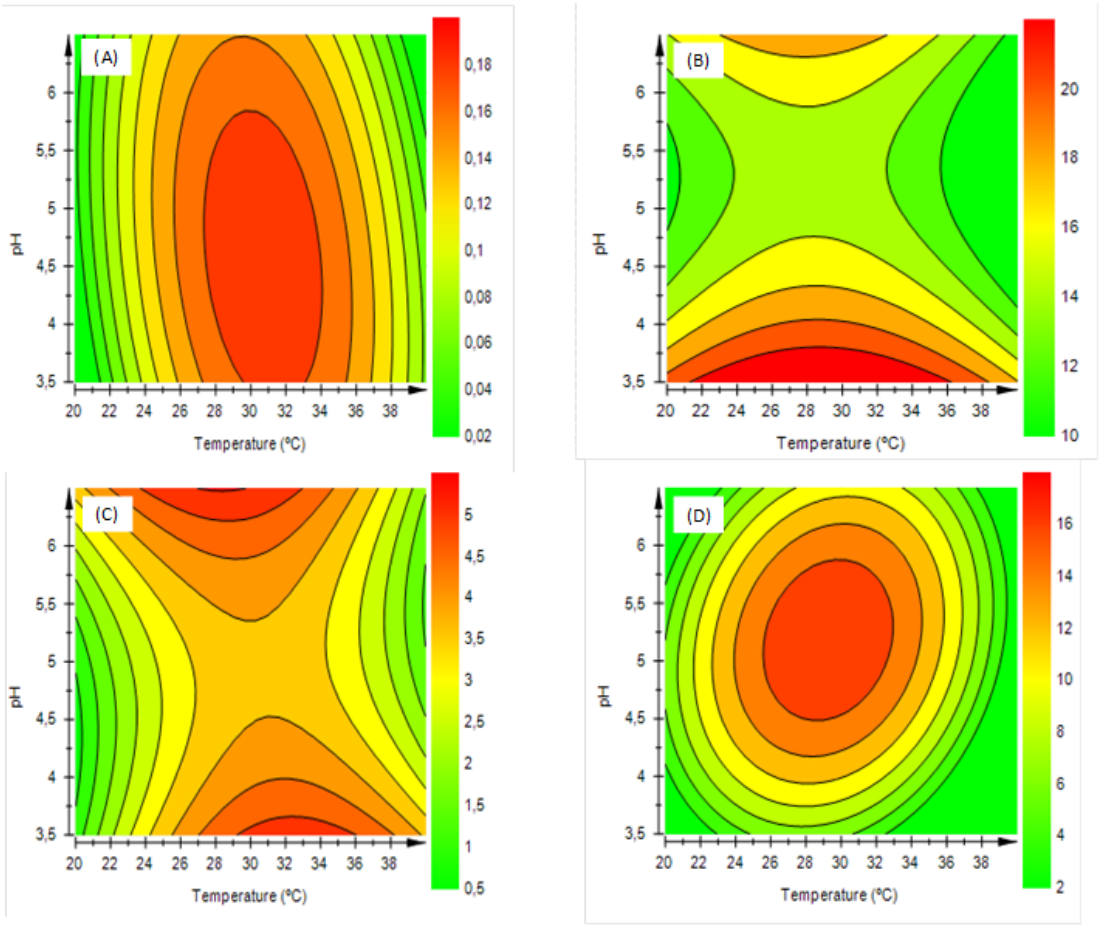

Figure 4.2 - Response surface of the responses studied as a function of pH and temperature of cultivation (maximum specific cell growth rate (A), CGC content in the biomass (B), volumetric productivity (C) and polymer´s chitin:β-glucan molar ratio (D)).

The knowledge and control of the cell wall composition of *K. pastoris* could be important for biotechnological purposes, due to the recent increasing commercial interest in the production of CGC. It can also be useful to obtain CGC with distinct chitin:β-glucan molar ratios that may impart the polymer different physical properties.

4.5 Conclusions

Response surface models were able to describe the interactive effect of temperature and pH on CGC production by *K. pastoris* and on the polymer's composition. Cultivation of *K. pastoris* within 27-33 ºC maximized both the CGC content in the biomass and the polymer's chitin:β-glucan molar ratio. In contrast, the pH ranges were not coincident for both responses. Thus, a compromise must exist between maximal CGC production (obtained for pH below 4.0 or above 6.0) and the synthesis of polymers enriched in chitin (obtained for pH = 4.5–5.8).

Chapter 5

Effect of Medium Composition on CGC Production

5.1 Summary

This study aimed at investigating the effect of different carbon sources, micronutrients content and composition, and the presence of toxic or stimulatory compounds on CGC production by *Komagataella pastoris*, as well as the effect of the tested conditions on yeast cell wall composition, in terms of chitin:β-glucan molar ratio.

Shake flask assays were performed to evaluate the effect of: 1) different carbon sources, namely, lactose, sucrose and galactose; 2) different concentrations of glycerol (40 g/l, 60 g/l, 80 g/l and 100 g/l); 3) increased concentration of divalent cations, namely, Ca^{2+}, Mn^{2+} and Mg^{2+}; 4) presence of glucosamine; and 5) presence of caffeine.

The results showed that several factors affected *K. pastoris* growth and CGC content: increasing glycerol concentration from 40 to 60 g/l stimulated cell growth from 9.23 g/l to 10.26 g/l, respectively) and increased the biomass content in CGC from 15 to 22%. Increasing the concentration of $MgSO_4$, $CaSO_4$ and $CaCl_2$ had different influence on yeast cell growth. Supplementation with $MgSO_4$ and $CaCl_2$ led to a decrease of yeast cell wall (6.95 g/l and 4.36 g/l, respectively), while supplementation with $CaSO_4$ stimulated cell growth (12.43 g/l), compared to standard conditions (9.23 g/l), and it also led to higher CGC content in the biomass (27, 24 and 23 wt%, respectively. On other hand, increasing the concentrations of $MnCl_2$, shows no impact on yeast cell growth (9.29 g/l) as well on CGC content on biomass (16 wt%), comparing with standard conditions (9.23 g/l and 15 wt%, respectively).

Furthermore, the presence of caffeine or glucosamine in the cultivation medium increased the chitin:β-glucan molar ratio in the polymer from 16:84 mol%, obtained under the standard conditions (40 g/l glycerol), to 19:81 and 23:77 mol%, respectively.

Further experiments using food industry byproducts, namely, cheese whey, sugarcane molasses and spent coffee grounds hydrolysate, were also performed in shake flask in order to assess their potential valorization into CGC.

Among them, the ones which support cell growth and improved CGC production, namely, sugarcane molasses and spent coffee ground hydrolysate, were selected and tested in bioreactor experiments. Results demonstrated that, beyond glycerol, *K. pastoris* is able to use several carbon sources. Higher cell densities were achieved with sugarcane molasses (17.78 g/l) with a CGC content of 17.53 % that corresponds to a CGC concentration of 3.12 g/l.

K. pastoris yeast was found to vary in its ability to use the supplied carbon sources. However, glycerol byproduct at concentrations of 40 and 60 g/l, as well as supplementation with 200 mM of $CaSO_4$ were found to be the most suitable carbon sources for higher cell growth and to increase the CGC content in the biomass. Supplementation with glucosamine proved to increase the CGC content on biomass, as well as the chitin:β-glucan molar ratio.

5.2 Introduction

Optimized growth medium plays an important role on the production of biomass and target products. Trace salt solution, the amount and type of nitrogen and carbon sources, affect the amount of biomass, as well as the target products produced by the cells. Growth and production of metabolites by an organism in a bioprocess are affected by interactions between intracellular and extracellular factors (Serio et al., 2003).

Culture Medium

The first step of medium development is to decide on the necessary components and their concentration in the medium. These nutrients contain the chemical elements which constitute the cellular materials and structures, and are required for membrane transport, enzyme activity, and generation of energy (Scragg, 1988). One of the important medium components is the nitrogen source. In the culture medium, ammonium hydroxide solution is mainly used, which also has the effect of controlling the pH at the desired level (Cos et al., 2006).

Nutrients required for growth can be classified in two categories. The first one is macronutrients, generally needed in concentrations larger than 10^{-4} M, for example carbon, oxygen, nitrogen, hydrogen, sulfur, phosphorus, magnesium and potassium. The second one is micronutrients, needed essentially for growth in the concentrations of less than 10^{-4} M, such as trace elements (e.g. Ca^{2+}, Cu^{2+}, Fe^{2+}, Na^{+}, Mn^{2+}, Mo^{2+}, Zn^{2+}, and vitamins, etc). These micronutrients are added to the culture medium as mineral salts (Fiecher et al., 1984).

Manganese is an essential trace element at a concentration of 2–10 μM for optimal yeast growth, because it is integrated in some enzymes, such as pyruvate carboxylase, glutamine synthetase, and arginase (Wedler, 1994) and it is regulate the bud growth. Manganese enhances yeast growth, especially in aerobic conditions (Berg and Tymoczko, 2002). Stehlik-Tomas et al. (2004) reported Mn^{2+} had an important role in the metabolism of *S. cerevisiae* and showed that if Mn^{2+} ions were present in optimal concentrations (0.1 g/l) in the medium, the biomass growth was higher (6.3 g/l), compared to conditions without addition of manganese (~ 4.5 g/l) .

Magnesium is widely considered as an essential trace element, normally supplied in growth media at levels up to 50 ppm (Andrade et al., 2003). It is a structural component of ribosomes and it has numerous biochemical functions. In contrast, calcium the other main alkaline earth metal is known to have only a few biochemical functions and many cells actively release this element (Saltukoglu and Slaughter, 1983).

Generally, basal salt medium (BSM) and trace salts medium (PTM) are used for high cell density fermentation of *K. pastoris*. This is considered as a standard medium, though it may not be optimal for some target products, and may have some important problems, such as unbalanced composition, precipitation and high ionic strength, changing the effective concentration of the dissolved minerals in the medium, as well as turbidity, thus compromising cell density assessments (Cereghino et al., 2002; Cos et al., 2006). Modification of the standard BSM (Brady et al., 2001; Thorpe and D´Anjou, 1999; Jungo et al., 2006) and the influence of metals on both cell growth (GS115 strain) and product yield (β-galactosidase) in *K. pastoris* expression systems have been investigated in detail by Plantz et al. (2007). They found that the levels of metals reported by Stratton et al. (1998) are, in two orders of magnitude, in excess for the production of β-galactosidase. Moreover, Ghosalkar et al. (2008) optimized a level of salts, trace metals and vitamins for the growth of recombinant *P. pastoris* in culture using glycerol as the main carbon source. Therefore, the study of the potential effect of medium requirements, such as micronutrients content, on both cell growth and product yield of *K. pastoris* were evaluated on this chapter.

One of the important components in medium design for fermentation of *K. pastoris* is the carbon source, which plays an important role on cell growth. Most commonly used carbon sources for *K. pastoris* are methanol, glycerol, sorbitol, glucose, mannitol, and trehalose (Brierley et al., 1990; Sreekrishna et al., 1997; Thorpe et al., 1999; Inan and Meagher, 2001-b).

Culture medium optimization based on using low cost substrates allows increasing the economic viability of biopolymers production. In order to eliminate the dependence on a single substrate (pure or crude glycerol) and increase productivity and evaluate the impact on the CGC composition, the search for new carbon sources for this bioprocess could make the process more versatile. Alternatively to the biodiesel byproduct, several wastes/byproducts can be used as substrates for microbial growth, such as, for example, sugarcane molasses, cheese whey and spent coffee grounds.

5.2.1 *Wastes/byproducts as* Carbon Sources

In fermentation processes, the use of a culture medium based on low cost substrates allows increasing the economic viability of biopolymers production. Similarly to other biopolymers, the use of renewable materials for the production of CGC has a great interest.

This study aimed to assess the suitability of different substrates for the cultivation of the yeast *K. pastoris* and production of CGC. The first part of the work consisted in investigating the ability of the culture to use glucose, lactose, galactose and sucrose for growth and their impact on CGC production. In the second part of the study, the potential of different industrial wastes and/or byproducts as substrates for this yeast was evaluated, to finding substrates alternative to glycerol. The utilization of alternative substrates allows increase the process versatility, reducing the dependence on a single substrate and decreasing the production cost, since the price of glycerol (even from biodiesel industry) can vary widely and also lead to a variation of cost of CGC production.

5.2.1.1. *Cheese whey*

Cheese whey is an abundant residue/byproduct obtained during cheese processing and its disposal in the environment causes several problems. Because of the high volumes produced and its high lactose content (corresponding to a biochemical oxygen demand (BOD) of 30.000-50.000 ppm and a chemical oxygen demand (COD) of 60.000-80.000 ppm, cheese whey requires treatment prior to its disposal (Koller et al., 2008). The bioconversion of this by-product into added-value products is an important alternative to overcome this environmental problem.

Whey is the major by-product from the manufacture of cheese or casein bovine milk, representing 80 to 90% of the volume of milk transformed (Ahn et al., 2000) and retaining 55% of milk nutrients (Siso, 1996). Cheese whey is rich in fermentable nutrients such as lactose, lipids and soluble proteins (Table 5.1), and also contains citric acid, non-protein nitrogen compounds (urea and uric acid) and B group vitamins (Siso, 1996).

Lactose may be used as a carbon source for bacterial cultivation in the production of bioplastics (Lee et al., 1997) and ethanol and methane (Guimarães et al., 2010) that can be used as a source of energy. Other products, such as agricultural fertilizers, animal feeding supplements, organic

acids, vitamins, pharmaceutical products and supplements for baby milks can also be obtained by treatment of whey (Siso, 1996).

Whey proteins have high nutritional potential and high added commercial value (Zydney, 1998). These proteins can be used as simple protein supplements, for the manufacture of transformed food products because of their functional and technological characteristics. According to the procedure used for casein precipitation, the cheese whey produced can be acid (pH<5) or sweet (pH 6-7) (Siso, 1996).

Table 5.1- Composition of cheese whey (Siso, 1996).

Nutrient	**Concentration (% w/v)**
Lactose	4.5 – 5
Soluble Proteins	0.6 – 0.8
Lipids	0.4 – 0.5
Mineral Salts (sucha as NaCl, KCl and calcium salts)	8 – 10[(1)]
Lactic Acid	0.05

[(1)] Concentration on percentage of dried extract.

In opposition to sugarcane molasses, the yeasts that ferment lactose are rather rare, including *Kluyveromyces lactis*, *Kluyveromyces marxianus*, and *Candida pseudotropicalis* that ferment lactose to produce ethanol (Guimarães et al., 2010). Furthermore, the low yield of the fermentation of lactose by *K. pastoris* has been reported by Shahidan et al. (2011). In addition, production of CGC by *K. pastoris* yeast, using pure lactose or cheese whey as carbon source was never reported. Furthermore, cheese whey is a complex substrate, rich in other nutrients besides lactose (e.g. vitamins, proteins) that could function as growth enhancers.

5.2.1.2. *Sugarcane Molasses*

Molasses is a viscous and dark liquid, constituting a common side stream of sugarcane industry that remains after the removal of crystalline sucrose from the sugar liquor (Olbrich, 2006). Molasses can be commercialized at up to half of the price of pure sugars like glucose (Zhang et al., 1994).

Molasses contain sucrose (32%) as the major carbohydrate beside other sugars (glucose (14%), fructose (16%)) and additional components like various B-vitamins (thiamine, riboflavin, niacin, pyridoxine, biotin and folic acid) and numerous minerals (potassium, phosphate, calcium, magnesium, cupper, and iron) that can act as promoters for microbial growth (Veana et al., 2014; Leeson and Summers, 2000; Olbrich, 2006).

A vast number of microorganisms is able to metabolize sugarcane sucrose or at least its hydrolysis products, namely equimolar mixtures of glucose and fructose. In contrast, the utilization of other disaccharides like lactose that also occurs in large quantities as an industrial by-product, but, in contrast to sucrose, is only converted by a rather restricted number of microorganisms (Koller et al., 2007).

In principle, industrially relevant producers from sucrose can be divided into two distinct groups: the first directly hydrolyzes sucrose by existing intra- or extracellular enzymes (activity of invertase) and converts the hydrolysis products glucose and fructose for generation of catalytically active cell mass. The second group encompasses those microbial strains able to convert the monomeric sugars glucose and fructose, but do not possess the metabolic prerequisites for sucrose hydrolysis; in this case, enzymatic or chemical hydrolysis of sucrose prior to the bioprocess is required.

Furthermore, Shahidan et al. (2011) reported the utilization of this substrate to produce lipase by *K. pastoris* and Bhosale and Gadre (2001) reported the utilization of sugarcane molasses to produce β-carotene by *Rhodotorula glutinis*. In addition, this substrate has been employed as animal feed, baking, fuel, paper and cardboard procurement and ethanol, lactic acid, citric acid, sorbitol productions, among others (Veana et al. 2014). Moreover, production of CGC by *K. pastoris* yeast, using the variety of sugars present in sugarcane molasses as carbon source was never reported.

5.2.1.3. Spent Coffee Grounds

Spent coffee grounds (SCG) are the main solid residue obtained during the treatment of coffee powder (instant coffee preparation) with hot water or steam, with a worldwide annual production of 6,000,000 tons (Tokimoto et al., 2005). SCG is normally released to the environment, despite its toxic character conferred by caffeine, tannins and polyphenols, makes it a dangerous residue to the environment (Silva, 1998; Arce, 2009); used as fuel in industrial boilers of the same industry, due to its high calorific power or as animal feed, however due this high lignin content (25% w/w) was considered a limiting factor for this last application (Mussato, 2011; Tokimoto, 2005; Cruz, 1983). Despite this, new economical and environmental alternatives for SCG use as value added product are necessary.

For 1 ton of green coffee 650 Kg of SCG was generated and about 2 Kg of wet SCG are obtained for each 1 Kg of soluble coffee produced (Mussato et al., 2011a). The chemical composition of this residue reveals a high sugar content, particularly frutose and galactose (Table 5.2). The high content in sugar allows integration of SCG in cultivation medium for microorganisms, such as *S. cerevisiae* and *Pichia stipitis*. The hydrolysate resulting from the acid hydrolysis of SCG has been used in the fermentation medium by these yeasts for ethanol production, with 50.1 % and 51.9 % efficiency, respectively (Mussato et al., 2012).

Table 5.2- Chemical composition (g 100/g) of spent coffee grounds hydrolysate (Mussato et al., 2011b).

Spent Coffee Grounds Hydrolysate (%)						
Sugars						
Arabinose	Glucose	Frutose	Galactose	Ash	Water	Nono-sugars
1,7	8,6	21,2	13,8	1,6	36,7	15,8

Considering the variety of sugars present in spent ground coffee wastes (Mussato et al., 2011b), its suitability to produce CGC by *K. pastoris* yeast was assessed. To the best of my knowledge, production of CGC from spent ground coffee wastes was never reported.

5.3 Materials and methods

5.3.1. Yeast strain and medium

All experiments were performed with *Komagataella (Pichia) pastoris* strain DSM 70877. Pre-inocula and inocula for the experiments were prepared as described in chapter 2- section 2.3.
Aiming at optimizing the culture medium, the influence of carbon source (glucose, lactose, sucrose or glycerol), stimulatory/inhibitory factors (caffeine and glucosamine) and metal ions (manganese, magnesium and calcium salts) on *K. pastoris* growth and CGC content and composition were investigated. BSM was supplemented with one of each component, prepared separately, to give a glycerol concentration of 40 g/l or the different desired concentrations of glycerol as carbon source.

5.3.2. Shake Flask Screening

The experiments were performed in 500 ml shake flasks containing 150 ml of BSM medium, supplemented with the appropriate substrate and/or with inhibitory/stimulatory biomass growth factor (Table 5.3).

Table 5.3- Composition of components used in shake flask experiments.

Assay	Carbon source
1	Glycerol 40 g/l
2	Glycerol 50 g/l
3	Glycerol 60 g/l
4	Glycerol 100 g/l
5	Galactose 40 g/l
6	Lactose 40 g/l
7	Sucrose 40 g/l
8	Glycerol 40 g/l + Glucosamine 12 mM
9	Glycerol 40 g/l + Caffeine 12 mM
10	Glycerol 40 g/l + $MgSO_4$ 140 mM
11	Glycerol 40 g/l + $MnCl_2$ 200 mM
12	Glycerol 40 g/l + $CaSO_4$ 200 mM
13	Glycerol 40 g/l + $CaCl_2$ 200 mM

In each experiment, the medium was inoculated with 10% (v/v) of *K. pastoris* biomass, prepared as described above, and growth occurred at 30 ºC and 200 rpm. The initial pH was 5.0 and it was monitored throughout the runs.

The assays were run for 96 hours, and 3 ml samples were periodically taken for measurement of the optical density at 600 nm (OD600nm) and pH, and determination of the dry cell weight. At the end of the experiments, 20 ml samples were also collected for CGC extraction, and for quantification and polymer composition analysis using the procedures described in chapter 3.

5.3.2.1. Effect of different carbon sources

The substrate solutions (20 ml) were prepared individually: 0.3 g/ml lactose (Scharlau); 0.3 g/ml galactose (Fluka); 0.3 g/ml Sucrose (Fluka); and 6.0 g, 7.5 g, 9.0 g and 15.0 g of glycerol from the biodiesel industry (supplied by SGC Energia, SGPS, SA, Portugal, with a glycerol content of 86%) were autoclaved at 121 ºC for 20 minutes. Each solution was added to BSM medium (130 ml) at the time of inoculation to give the appropriate total sugar concentration.

5.3.2.2. Effect of of inhibitory/stimulatory growth factor

The 0.017 g/ml g/l caffeine (Sigma) and 0.016 g/ml glucosamine (Sigma) solutions (20 ml) were prepared individually in deionised water and autoclaved at 121 ºC for 20 minutes. Each solution, in the desired concentration (12 mM), was added to BSM medium (130 ml) at the time of inoculation.

5.3.2.3. Effect of Supplementation with Inorganic Salts

Concentrated inorganic salts solutions, 0.26 g/ml $MgSO_4$. 7 H_2O (LabChem), 0.19 g/ml of $MnCl_2$ (Acros), 0.20 g/ml $CaSO_4$ (Sigma) and 0.17 g/ml $CaCl_2$ (Panreac) were prepared individually in deionised water (20 ml), autoclaved at 121 ºC for 20 minutes and added to BSM medium (130 ml) at the time of inoculation, to obtain 140 mM $MgSO_4$. 7 H_2O, and 200 mM of $MnCl_2$, $CaSO_4$ and $CaCl_2$ on BSM medium.

5.3.2.4. Waste Substrates Preparation

The cheese whey used in this study was supplied by Lactogal (Portugal). The cheese whey powder (6 g) was dissolved in deionised water (20 ml). The solution was autoclaved at 121 ºC for 20 minutes and centrifuged (17.418 × *g* for 15 minutes) to remove precipitated protein aggregates and added to BSM (130 ml) at the time of inoculation, to obtain the desired concentration of 40 g/l.

The sugarcane molasses used in this study were supplied by RAR – Refinarias de Açúcar Reunidas, Portugal. The sugarcane molasses (12 g) were diluted in deionised water (20 ml). The solution was sterilized at 121 ºC for 20 minutes and added to BSM (130 ml) at the time of inoculation, to obtain the pretended concentration (80 g/l).

The spent coffee grounds were subjected to acid hydrolysis prior to their use in the experiments. Spent coffee grounds were supplied by the Cafeteria of the Chemistry Department, at FCT-UNL. Before hydrolysis, the spent coffee grounds were dried (70 ºC for 3 hours). To prepare the solutions for hydrolysis, 10 g of spent coffee grounds were mixed with 100 ml of deionised water. Acid hydrolysis was performed with sulfuric acid 98% (H_2SO_4), in a concentration of 100 ml/l, at 100 ºC during 45min. After cooling to room temperature, the hydrolysates were centrifuged (9820 × *g*, for 30 minutes) and two fractions were obtained: a solid residue and a sugar solution. The solid residues were discarded and the sugar solution was used for the

experiments. Before their use, the pH of the hydrolysates was adjusted to 5.0 by the addition of a 25% (v/v) ammonium hydroxide solution.

5.3.3 Bioreactor operation

All experiments were performed with *Komagataella (Pichia) pastoris* strain DSM 70877. The influence of different raw materials used as carbon source (sugarcane molasses and coffee spent grounds hydrolysate) on *K. pastoris* growth , CGC content on biomass and chitin:β-glucan molar ratio were investigated.

Inocula for bioreactor experiments were prepared by incubating the culture in BSM medium, containing 100 g/l of spent coffee grounds hydrolysate or 80 g/l of sugarcane molasses as carbon sources, in shake flasks for 3 days at 30 ºC, in an incubator shaker (200 rpm). 10% vol/vol inocula were used to inoculate the 5 l bioreactor (BioStat B-plus, Sartorius), which was operated at 30 ºC. pH was controlled at 5.0 by the automatic addition of 25% (v/v) ammonium hydroxide solution that served also as the nitrogen source. The dissolved oxygen (DO) concentration was controlled at 50% air saturation by the automatic variation of the stirring rate (between 300 and 2000 rpm) and supplementation of the air stream with pure oxygen. All experiments were performed in a batch mode. Samples (20-25 ml) were periodically withdrawn from the bioreactor during the experiments for determination of the cell dry weight, CGC content in the biomass, polymer composition and .

5.3.4. Analytical techniques

CDW and nutrients concentration, as well as CGC content and composition were determined as described in section 4.3.3. – Chapter 4.

5.3.5. Substrate Concentration

For quantification of sugars, cell-free supernatant samples collected during the experiments were diluted. After dilution, the samples were filtered with 0.20 μm centrifuge filters (9 600 × g for 5 minutes; VWR) prior to the analysis.

The concentration of sugar monomers on wastes/byproducts substrates (lactose, galactose, glucose, fructose, sucrose, mannose and arabinose) were determined by HPLC using a CarboPac PA10 column (Dionex), equipped with an amperometric detector. The analysis was performed with sodium hydroxide (NaOH 18 mM) as eluent, at a flow rate of 0.8 mL min^{-1} and carried out at 30 ºC. For this method, samples were dilutes with deionised water. Lactose, galactose, glucose, fructose, sucrose, mannose and arabinose (Sigma-aldrich) with 0.006 - 0.2 g/l, were used as standards.

For the concentration of the total sugars in spent coffee grounds hydrolysate and sugarcane molasses samples, Dubois method was used. Dubois is a colorimetric method where 2.5 ml of H_2SO_4 was added to the 0.5 ml of 5% (v/v) phenol solution and 0.5 ml of sample. The solution was kept in the dark for 30 minutes and its optical density was measured at 490 nm. In this method, glucose solutions with concentrations of 0.003 - 0.1 g/l were used as standards.

5.3.6. Kinetic parameters

The maximum specific cell growth rate (μ_{max}, h^{-1}) was determined as described in section 2.3 – chapter 2. The CGC volumetric productivity (r_p, gCGC/l·day) was determined as follows:

$$r_p = \Delta p/\Delta t \qquad (1)$$

$$\Delta p = CDW \times \%CGC \qquad (2)$$

where Δp corresponds to the product, CGC (g/l), produced within time interval Δt (day), and %CGC is the CGC content in the biomass (wt%).

5.4 Results and Discussion

5.4.1 Effect of Increasing Glycerol Concentration

The impact of increasing glycerol concentration (40, 50, 60 and 100 g/l) on *K. pastoris* growth, production of CGC and chitin:β-glucan molar ratio was tested in four batch shake flask experiments.

Under the batch conditions (assays 1 to 4, Table 5.4), it was observed that cell growth was slightly increased from 9.23 g/l using 40 g/l (assay 1) to 10.26 g/l by cultivation with BSM supplemented with glycerol at a concentration of 60 g/l. No significant change on cell growth by cultivating *K. pastoris* with 40 or 50 g/l (9.74 g/l). Higher glycerol concentration (100 g/l) reduced cell growth to 6.22 g/l. On the other hand, the highest CGC content (> 15%, w/w) in *K. pastoris* biomass was obtained for 60 (22 %, w/w) and 100 g/l glycerol (23 %, w/w). Osmotic pressure has been known to exert deep influence on cell growth and on its metabolism. High osmotic pressure is a key factor responsible for the inhibition of yeast growth and decrease fermentation performance under high substrate concentrations (Puligundla et al., 2011). Thereby, a glycerol at 60 g/l is the best concentration since produce high biomass concentration (10.26 g/l) with high content of CGC (22 % w/w) and with a similar chitin:β-glucan molar ratio (13:87), comparatively to standard conditions (16:84; assay 1, Table 5.4).

Table 5.4- *Komagataella pastoris* batch shake flask assays, under different cultivation conditions.

Assay	Carbon source	CDW (g/l)	CGC (wt%)	chitin:glucan ratio (mol%)
1	Glycerol 40 g/l	9,23	15	16:84
2	Glycerol 50 g/l	9,74	13	10:90
3	Glycerol 60 g/l	10,26	22	13:87
4	Glycerol 100 g/l	6,22	23	13:87
5	Galactose 40 g/l	2,81	14	15:85
6	Lactose 40 g/l	2,53	21	19:81
7	Sucrose 40 g/l	3,64	23	19:81
8	Glycerol 40 g/l + Glucosamine 12 mM	7,99	17	23:77
9	Glycerol 40 g/l + Caffeine 12 mM	3,42	25	19:81
10	Glycerol 40 g/l + $MgSO_4$ 140 mM	6,95	27	13:87
11	Glycerol 40 g/l + $MnCl_2$ 200 mM	9,29	16	11:89
12	Glycerol 40 g/l + $CaSO_4$ 200 mM	12,43	24	15:85
13	Glycerol 40 g/l + $CaCl_2$ 200 mM	4,36	23	10:90

5.4.2 Screening of different sugars as substrates (galactose, lactose, sucrose) for cultivation of K. pastoris and CGC Production.

The aim of this study was to assess the ability of *K. pastoris* for utilization of pure lactose, galactose and sucrose, as carbon sources. Hence, each of these substrates was tested in batch shake flask experiments to assess their suitability for *K. pastoris* cultivation and production of CGC. Table 5.4 shows the results obtained in each assay in terms of CDW, CGC content and chitin:β-glucan molar ratio.

All tested carbon sources considerably depressed cell growth: the use of galactose, lactose and sucrose as carbon sources resulted in low CDW values of 2.81, 2.53 and 3.64 g/l, respectively. These values are lower than those obtained with glycerol 40 g/l (9.23 g/l). These results are in accordance with González-Barroso et al. (2006) that reported the inability of *P. pastoris* to use galactose and lactose as sole substrate. Comparing to CGC obtained with standard conditions (15 wt%), the highest CGC content in *K. pastoris* biomass was obtained for cultivation with BSM supplemented with lactose (21 wt%) or sucrose (23 wt%) as carbon sources. Supplementation with galactose at 40 g/l slowly decreased (14 wt%) the CGC content on the biomass. Similarly, both BSM supplementation with lactose or sucrose equally increased chitin:β-glucan molar ratio (19:81), and supplementation with galactose slightly decreased chitin:β-glucan molar ratio (15:85), comparatively to standard conditions (16:84; assay 1, Table 5.4).

K. pastoris is known as Crabtree-negative species, metabolizing sugars preferentially through the respiratory circuit, which is a major reason for the easy growth to a high cell density. Under these shake flask assays conditions, yeast growth and metabolism are mainly affected by the difficulty in metabolizing the carbon source, which might justify both increasing the CGC content on the biomass, as well as on chitin:β-glucan molar ratio. There is always a long lag phase after inoculation, which is followed by a short cell proliferation step. Results showed that *K. pastoris* cannot use sucrose as the sole carbon source (Sreekrishna et al., 1987). Moreover, the observed growth delay may be related with sucrose slower degradation, due the fact that it is a disaccharide. Furthermore, the prior synthesis of enzymes (invertase enzyme) to sucrose hydrolysis, resulting an equimolar mixture of fructose and glucose, increased energy expenditure focused to other metabolic pathways and also translated in a growth delay.
Furthermore, *K. pastoris* was also unable to metabolize lactose, as shown by the reduced cell growth (2.53 g/l). These results could be justified by two reasons: a) do not have the ability to import lactose due to the absence of lactose permease; b) does not have the enzyme β-

galactosidase required to break lactose into its constituent sugars, galactose and glucose. The enzymes that process galactose and glucose are unable to process lactose directly (Ramakrishnan and Hartley, 1993).

Shahidan et al. (2011) reported a low yield of the fermentation of lactose by *K. pastoris.* However, production of CGC by *K. pastoris* yeast, using pure lactose as carbon source was never reported. In this assay, CGC content in the biomass was evaluated. Indeed, increased CGC content in the biomass (21 %) and higher chitin:β-glucan molar ratio (19:81) were obtained, comparatively to standard conditions (15% and 16:84; assay 1, Table 5.4), respectively. Thus, although the lower cell growth observed, lactose presents a promising source to obtain CGC enriched in chitin. In this sense, these results provide a promising alternative for production of CGC using food byproducts rich in lactose, such as cheese whey.

5.4.3 Effect of Glucosamine

In order to determine the effect of glucosamine on *K. pastoris* cultivation, a shake flask screening was performed (assays 8, Table 5.4).

Supplementation with glucosamine depressed cell growth (7.99 g/l), comparatively to the value obtained with standard conditions (9.23 g/l). Nonetheless, compared to standard conditions (15 wt%), glucosamine slightly stimulated CGC content on biomass (17 wt%). Data from assay 8, revealed that the presence of glucosamine apparently enriched the biomass in CGC and in chitin, as shown by the higher chitin:β-glucan molar ratio (23:77 mol%), comparatively with standard conditions (assay 1, 16:84). Other authors, such as Bulik et al. 2003 showed that the presence of glucosamine on medium increased in three to four-fold cell wall chitin contents.

5.4.4 Effect of Caffeine

Supplementation with caffeine on *K. pastoris* cultivation was evaluated in a shake flask assay (assay 9, table 5.4). Kuranda et al. (2006) reported that the presence of caffeine affected *Saccharomyces cerevisiae* cell growth and caused cell wall damage. Indeed, a much lower cell growth value (3.42 g/l) was obtained, compared to the standard cultivation value (9.23 g/l), demonstrating that caffeine has been a stress growth factor for *K. Pastoris*. In contrast, increased CGC contents on biomass (25 wt%) and chitin:β-glucan molar ratio (19:81) were

obtained, comparatively with standard conditions (15 wt% and 16:84, respectively). Hence, the higher CGC content and the increased chitin:β-glucan molar ratio might be related to the defense mechanism against the presence of chemical agent (caffeine) that induced cell wall stress.

5.4.5 Effect of Supplementation with Inorganic Salts

Generally, the addition of all investigated minerals (manganese, calcium and magnesium) improved sugar consumption and higher cell densities when compared to the fermentation under standard conditions, without the supplementation of inorganic salts (Ghosalkar et al., 2008).

Among all tested inorganic salts, alone, the most efficient in improving yeast cell growth were a supplementation with $MnCl_2$ (200 mM) or $CaSO_4$ (200 mM) (assays 11 and 12, Table 5.4) that resulted in increased cell growth (9.29 and 12.43 g/l, respectively), comparatively to standard conditions (assay 1, Table 5.4; 9.23 g/l). Positive effects were also obtained with the addition of $MgSO_4$, $MnCl_2$, $CaSO_4$ and $CaCl_2$ at concentrations between 140 and 200 mM, that resulted in high CGC contents in the biomass (between 16 and 27%). It can be hypothesized that the positive effect of divalent cation salts was due to a stimulatory effect of cations on CGC synthesizing enzymes. However chitin:β-glucan molar ratio was maintained between 10:90 and 15:85, which is below of standard conditions (16:84; assay 1; Table 5.4). Furthermore, the use of $CaSO_4$ or $CaCl_2$ as inorganic salts supplementation on BSM culture medium revealed a considerable variation on cell growth. The yeast cell growth was 4.36 g/l when calcium chloride was added to the basal medium and 12.43 g/l in the presence of supplementation with calcium sulphate.

Yeast growth in culture medium that was supplemented with calcium chloride was slower than that in the calcium sulphate (Table 5.4). Based on the above information, it can be hypothesized that supplementation of sulphate ions increased the growth of *K. pastoris* yeast.

This hypothesis is consistent with results obtained by Jayakody et al. (2013), wherein yeast cells are affected by deficiency of extracellular sulphate ions during growth. Supplementation with sulphate ions (8.5 mM), instead chloride ions or sodium ions, stimulated yeast cell growth.

Calcium also has an important role in the maintenance of the integrity of membranes and a possible function in enzymatic activity (Landecker, 1996), which could justify the increased of CGC content on biomass in these assays (24 and 23 %, respectively).

5.4.6. Valorization of food and industry byproducts for the production of CGC

Following the previous results, a shake flask screening was performed with the aim to select in order to valorize wastes/byproducts resulted from food and industry for use as alternative substrates for *K. pastoris* cultivation and CGC production. The screening was performed with cheese whey, which is rich in fermentable nutrients such as lactose, lipids and soluble proteins, lactose is the main sugar present in cheese whey wastes (4.5-5% w/v). The bioconversion of whey lactose into CGC is very interesting since it allows its valorization and reduce the environmental problem due to its disposal; sugarcane molasses which is composed mainly by sucrose (32%) as the major carbohydrate beside other sugars (glucose (14%), fructose (16%)) and additional vitamins and minerals.; and with spent coffee grounds hydrolysate with sulfuric acid, considering the variety of simple sugars present on hydrolysate that contained 35.66 g/l of monosaccharides, being constituted by 48.93% mannose, 39.40% galactose, 8.61% arabinose and 3.06% glucose. Mussatto et al. (2011b) reported, with slightly different values, the presence of mannose (46.8%), galactose (30.4%), glucose (19%) and arabinose (3.8%). The higher glucose concentration obtained by Mussatto et al. (2011b) may be related to the different acid hydrolysis conditions applied, namely higher temperature and longer reaction time (163 ºC for 45 minutes), which were necessary for complete hydrolysis of the cellulose fraction. Given that they are wastes/surplus inexpensive available in high amounts and are non-seasonal, which make the process economically and environmentally sustainable. The results on biomass growth using these feedstocks are shown in Figure 5.1.

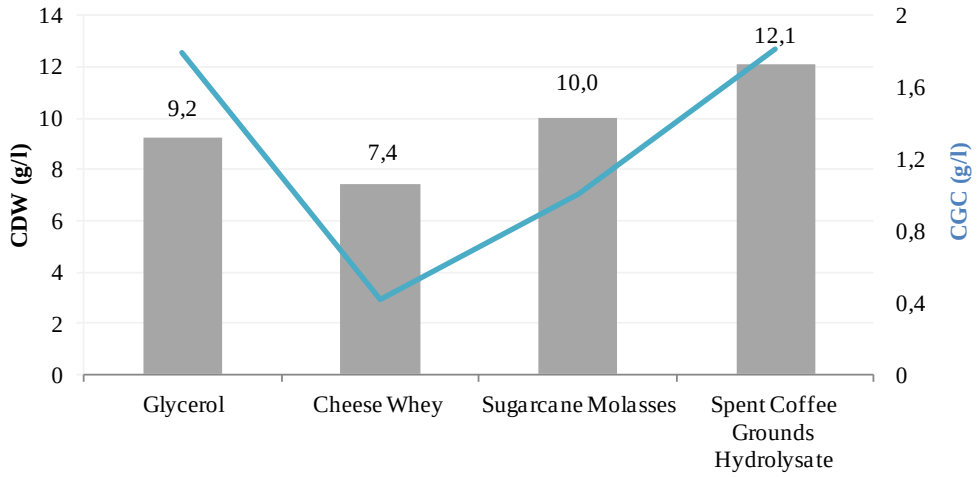

Figure 5.1- Biomass growth (CDW) and CGC content on biomass (g/l), obtained by cultivation of *K. pastoris* in the shake flask assays using cheese whey, sugarcane molasses and spent coffee grounds hydrolysate as carbon sources.

For the assays performed with cheese whey as carbon source, the culture achieved a biomass concentration of 7.4 g/l, a value lower than for sugarcane molasses (10.0 g/l) and spent coffee grounds hydrolysate (12.1 g/l) (Figure 5.1), these last two values were higher than those obtained with glycerol byproduct (9.23 g/l) under identical cultivation conditions. These results are in agreement with studies for *P. pastoris* strain GS115 growing in medium containing lactose (1%), where biomass growth was lower than that obtained with 1% of sugarcane molasses (Shahidan et al., 2011).

Mussatto et al. (2012) and Hugues et al. (2014) studied, in shake flask assays, the impact of using spent coffee grounds hydrolysate on growth of *S. cerevisiae* to ethanol production. The biomass growth (5.5 g/l) was slower than obtained in this study with *K. pastoris.* Despite this difference, it should be taken in account that this experiment was performed in a shake flask with yeast extract on the culture medium. However, spent coffee grounds hydrolysate is an agro-industrial waste with large potential for ethanol production, since interesting results of ethanol productivity were obtained when the sugar-rich hydrolysate produced from this material was used as fermentation medium by *S. cerevisiae*, comparatively to other tested residues (coffee silverskin hydrolysate) (Mussato et al., 2011).

Biomass concentration achieved with sugarcane molasses as carbon source (10 g/l) is similar to studies for the yeast *Rhodotorula glutinis* (6.6 –12.0 g/l), where sugarcane molasses were used to produce β-carotene, in shake flask experiments (Bhosale and Gadre, 2001). The utilization of molasses by *K. pastoris* strain GS115 has already been reported by Shahidan et al. (2011), but the biomass concentration achieved was not reported.

CGC was produced by *K. pastoris* in all of the 3 experiments analyzed (Figure 5.1). However, the CGC content in the biomass was not similar for the experiments performed with different carbon sources. The results obtained showed that the higher CGC content was obtained with glycerol as carbon source (1.79 g/l), but it decreased for cheese whey (0.42 g/l) and for sugar cane molasses (1.01 g/l) used as carbon sources. Concomitantly, CGC production was higher (1.01 g/l) in the assays with sugarcane molasses wherein higher biomass concentration (10 g/l) was also reached. In the experiments with cheese whey, concomitantly with the low cell growth observed (7.4 g/l), CGC production was also very low: 0.42 g/l. So, it appears that with an increase in biomass resulted in an increase on CGC production, with this results and despite not having been analyzed the CGC content on biomass resulted from assay with spent coffee grounds hydrolysate as carbon source, due its high CDW (12.1 g/l) a batch bioreactor experiment was performed with this substrate.

These results show that *K. pastoris* was able to consume the different tested residues, being their composition a determinant factor to yeast growth. This strain showed preference to residues rich in glucose in its constitution such as sugarcane molasses and spent coffee grounds hydrolysate, while cheese whey was not a suitable substrate. Based on these results, sugarcane molasses and spent coffee grounds hydrolysate were selected, among the tested residues, for further experiments with *K. pastoris* cultivation in bioreactor.

5.4.7. Batch Bioreactor experiments

Due to the higher performance of *K. pastoris* yeast in shake flask experiments with sugarcane molasses (80 g/l) and spent coffee grounds hydrolysate (100 g/l) as carbon sources, the latter were tested in batch bioreactor experiments.

The culture's performance in both experiments was compared with the cultivation of *K. pastoris* using glycerol from the biodiesel industry as sole substrate (chapter 4-section 4.4.1) (Table 5.4).

Table 5.5- Comparison of parameters obtained for different residues in batch bioreactor experiments. μmax, maximum specific growth rate; CDW, maximum cell dry weight; % CGC, % of CGC in dry cell biomass; CGC, maximum active concentration; r_p, overall volumetric productivity.

Residue	**Biodiesel Byproduct**	**Sugarcanne Molasses**	**Spent Coffe Grounds Hydrolysate**
Cultivation time (h)	48	71	92
μ_{max} (h^{-1})	0,17	0,11	0,051
CDW (g/l)	33,6	17,78	3,46
%CGC	13.0	17,5	15,0
r_p (g_{CGC}/l. h^{-1})	0,139	0,044	0,006

The cultivation profile of *Komagataella pastoris* on sugarcane molasses (Figure 5.2) showed that, after a 7 hours lag phase, *K. pastoris* grew at a specific cell growth rate of 0.11 h^{-1}, attaining maximum biomass concentrations of 17.78 g/l within 71 hours (Table 5.4), with a total sugar consumption of 20 g/l, as carbon source (Figure 5.2). This value was higher than that obtained for *Rhodotorula glutinis* (10 g/l) cultivated on molasses for β-carotene production in batch fermentation, reported by Bhosale and Gadre (2001). For *K. pastoris* wild type strains, there are no reported studies about its growth in sugarcane molasses.

A final CGC content of 17.53% in the biomass was reached at the end of the experiment, corresponding to a CGC concentration of 3.12 g/l (Table 5.4).

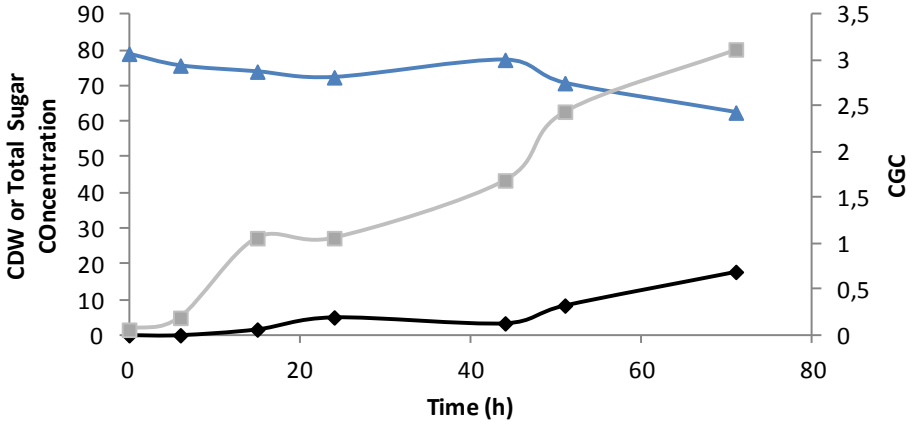

Figure 5.2- Cultivation profiles of the batch bioreactor experiments with sugarcane molasses. Dry cell weight (—, g/l) and CGC (—, g/l) and total sugars concentration (—, g/l).

Considering the time frame of increased CGC production (71 hours), a volumetric productivity (r_P) of 0.044 g/l. h^{-1} (Table 5.4) was achieved, a lower value than the 0.139 g/l. h^{-1} obtained using glycerol byproduct as carbon source in a batch reactor during 48 hours (Table 5.4). Furthermore, a CDW lower (17.78 g/l) than obtained with glycerol byproduct (33.6 g/l), consequently, did not result in a lower CGC amount. Despite those lower values, the CGC content in the biomass obtained for sugarcane molasses (17.53%) was higher than that obtained with glycerol (13%). As proposed by Ketela et al. (1999), it can be hypothesized that the lower growth could have caused stress for the cells and increased the production of CGC.

The experiment with spent coffee grounds hydrolysate (Figure 5.3-A) was also carried out in a 5l bioreactor fed with 100 g/l spent coffee grounds hydrolysate. All the other conditions were maintained unchanged. After a short adaptation period, *K. pastoris* started to growth and reached a maximum biomass concentration of 3.46 g/l. This value is five-fold lower than that obtained for growth with sugarcane molasses as carbon source (17.78 g/l). Kuranda et al.

(2006), reported that the presence of caffeine in coffee spent ground hydrolysates confers an inhibitory growth factor, since for *S. cerevisiae* caffeine inhibits the cellular growth by 50%, however is a stimulatory factor for CGC production, it induces the increase of the chitin content in the cell wall as a response to stress (Ketela et al., 1999).
As mentioned above, cultivation of *K. pastoris* in spent coffee grounds hydrolysates has still not been reported.

At the end of the experiment, cell concentration reached 3.46 g/l with a CGC concentration (0.52 g/l; Table 5.4) that represented 15.04% of biomass, which was lower than that achieved with sugarcane molasses (17.53 %). This difference could be justified by the lower sugar concentration available as carbon source (only 18 g/l of monosaccharides), resulting in a lower biomass concentration.
The volumetric productivity was also lower, reaching only 0.0057 g/l.h, comparatively to values obtained using sugarcane molasses (0.044 g/l h^{-1}) and glycerol byproduct (0.139 g/l.h) as carbon source (Table 5.4).
These results showed that performance of *K. pastoris* in BSM medium using coffee spent ground hydrolysate was less interesting than sugarcane molasses. Though, in this experiment, initial spent coffee ground hydrolysate monosaccharides concentration were not the same, as the time consumption it is not performed at the same time (figure 5.3-B). So, in this experiment, glucose was consumed preferentially for cell growth (specific growth rate of 0.051 h^{-1}) and its depletion occurred within the initial 16 hours of the experiment. Glucose is a favored carbon and energy source in yeasts (Weinhandl et al., 2014).

The simple monosaccharide glucose and fructose are the preferred carbon sources of the budding yeast, although when these sugars are not available yeast can utilize alternative carbon source such as sucrose (Gancedo, 1998), a disaccharide composed of glucose and fructose. Following glucose exhaustion, started mannose consumption and the culture continued to grow but at a lower specific growth rate (0.033 h^{-1}). Contrary, galactose and arabinose concentration increased during the experiment, this could be justified by the enzymatic hydrolysis of the oligo- and polysaccharides present in the hydrolysate, which produce more glucose and mannose that is rapidly consumed, while galactose and arabinose were accumulated in the broth. Furthermore, culture continued to grow after mannose depletion, again justified by hydrolysis of such oligo- and polysaccharides.

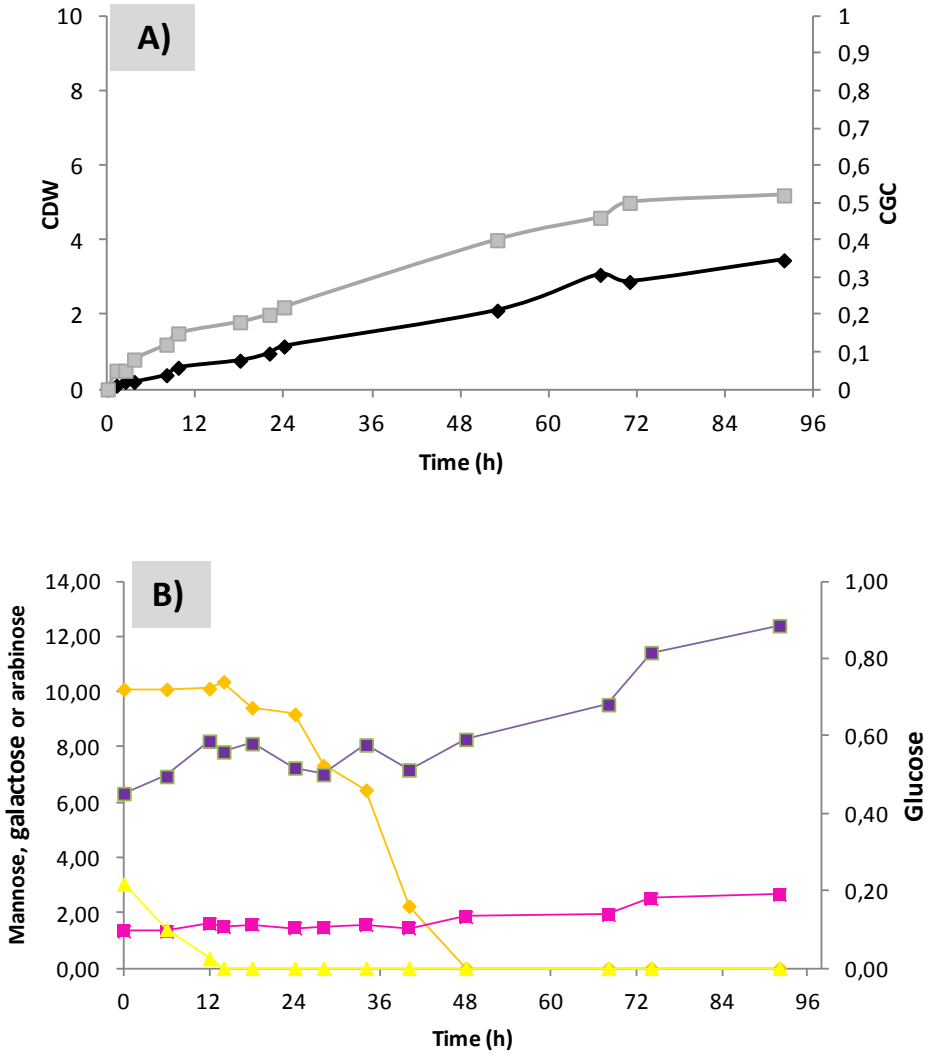

Figure 5.3- Cultivation profiles of the batch bioreactor experiments with spent coffee grounds hydrolysate: A - CDW (—, g/l) and CGC concentration (—, g/l); B - galactose (—,g/l), glucose (—, g/l), mannose (—, g/l) and arabinose concentration (—, g/l).

Due to the lower biomass concentration achieved in the experiments with sugarcane molasses and spent coffee grounds hydrolysate, CGC composition in glucans and chitin was not possible. In summary, spent coffee grounds hydrolysate used as carbon sources significantly decreased *K. pastoris* cell growth and low biomass concentration values at the end of both experiment (3.46 g/l), comparatively with the results obtained with glycerol from biodiesel industry.

5.5 Conclusions

In shake flask assays, appropriate(s) carbon source and inhibitory/stimulatory growth factors for *K. pastoris* cultivation and CGC production were investigated. Under shake flask conditions, it was observed that cell growth was higher for glycerol at concentrations of 40 g/l (9.23 g/l) and 60 g/l (10.26 g/l) as carbon source. Although the use of glycerol at 100 g/l, lactose and sucrose as carbon sources led to reduced cell growth, nonetheless the biomass had an increased CGC content (21-23 wt%), compared to a standard conditions (15 wt%).

Supplementation with $MnCl_2$ or $CaSO_4$ also resulted in increased cell growth. On the other hand, the use of galactose (30 g/l), lactose (40 g/l) or sucrose (40 g/l) as carbon sources, and supplementation with caffeine depressed cell growth (CDW < 4.00 g/l). Supplementation of BSM with caffeine and glucosamine also resulted in high CGC content (25 and 17%, w/w, respectively).

Several wastes and/or byproducts (sugarcane molasses, cheese whey and spent coffee grounds hydrolysate) were tested. In batch bioreactor, the best results were achieved with sugarcane molasses, where 17.78 g/l biomass contains 17% of CGC, a value higher than that obtained with glycerol byproduct (16%). On the other hand, biomass concentration obtained using coffee spent ground hydrolysate was much lower (3.46 g/l) than with sugarcane molasses and glycerol biodiesel byproduct (33.6 g/l). The volumetric productivity obtained with coffee spent ground hydrolysate was also lower, reaching only 0.0057 g/l h^{-1}, comparatively to the values obtained using sugarcane molasses (0.044 g/l h^{-1}) and glycerol byproduct (0.139 g/l h^{-1}). These lower values are a consequence of the lower biomass concentration achieved with those wastes and indicate that they are not suitable for *K. pastoris* cultivation nor for CGC production. Moreover, due to the lower biomass concentration achieved in the experiments with sugarcane molasses and spent coffee grounds hydrolysate, it was not possible to analyze the CGC polymers for their composition in glucans and chitin. Evaluation of the yeast cell wall composition, in terms of chitin:β-glucans molar ratios is important to determine the polymer's possible applications, as already mentioned in chapters 1 and 3.

Chapter 6

Conclusions and Future Strategies

6. General Conclusions and Future Work

6.1. *General Conclusions*

In this thesis, *Komagataella pastoris* was cultivated using glycerol byproduct from the biodiesel industry aiming at producing CGC co-polymer. *K. pastoris* DSM 70877 was selected as a high biomass producing yeast strain in pure glycerol (99%, w/v) and in glycerol from the biodiesel industry (86%, w/v), in shake flask assays. Furthermore, final wet cell weight of 95 g/l within 79 hours, was achieved with *K. pastoris* DSM 70877 strain using glycerol byproduct (43 g/l) while 82 g/l was achieved using pure glycerol (40 g/l). These results may be related with the strain but also with the impurities presents on glycerol byproduct which might have contributed to improve the yeast cell growth.
K. pastoris DSM 70877 strain was cultivated in bioreactor in fed-batch mode using glycerol byproduct at concentration of 40 g/l. High cell density (104 g/l) was obtained in less than 48 h. The yield of biomass on a glycerol basis was 0.63 g/g during the fed-batch phase.

Significant amounts of CGC (14-79%) were found on the *K. pastoris* yeast cell wall, depending of extraction and purification procedure. The first procedure to extract CGC from the biomass consisted on using 1 M NaOH at 65 ºC, during 2 h, followed by sequential washing steps: water/PBS/ ethanol/water. Results showed that chitin:β-glucan molar ratio of CGC_{PBS} had lower values (16:84 mol%) than the values reported for kiOsmetine (between 30:70 and 50:50 mol%). This lower ratio of chitin:β-glucan was related to the extraction procedure used that resulted in an impure polymer likely containing alkali soluble glucans and mannose polysaccharides that were not completely removed during extraction and purifications steps. Indeed, the polymer had higher contents of mannose of CGC (28 wt%), total protein (9.5 wt%), and inorganic salts (15.0 wt%), than those from kiOsmetine (mannose of 1.7 wt%, reduced protein <8 wt%) and ash of <5 wt%) (www.kitozyme.com). In order to increase the degree of purity, further extraction and purification improvements were accessed. It was found that increasing the inorganic solvent extraction from 1 M to 5 M NaOH at 65 ºC, during 2 hours, associated to neutralization with HCl, followed repeatedly washed with deionised water until conductivity ≤20μS/cm increased CGC purity, improving the solubilization of proteins and alkaline soluble polymers, such as glucans and mannans.

A purified CGC was obtained from *K. pastoris* biomass, CGC_{pure}, with a molar ratio (25:75 mol%) that was similar to the values reported for kiOsmetine (30:70 mol%). Furthermore, residual contents of mannose (1.5 wt.%), proteins (3 wt.%) and inorganic salts (0.9 wt.%) were lower than the commercial CGC from Kitozyme (kiOsmetine).

DCS and NMR spectroscopy were used to compare CGC_{pure} with commercial biopolymers, such as chitin, laminarin and CGC (KiOsmetine from Kitozyme). DSC results showed distinct thermal properties, namely a single narrower endothermic peak, indicating the presence of minor proteins and the absence of salts, comparatively to the commercial CGC biopolymer (KiOsmetine). NMR results confirmed that CGC_{pure} contains less chitin than β- glucan monomers with respect to the commercial biopolymer from Kitozyme and ^{13}C resonance confirmed that purified CGC biopolymer is essentially a superposition of the commercial laminarin and the crab shell chitin ^{13}C spectra. Moreover, the ^{13}C spectrum of CGC_{pure} showed approximately the same resonance positions as the ^{13}C spectra of the commercial biopolymer obtained from Kitozyme, which indicates that both chemical structures are very similar.

Furthermore, *K. pastoris* was able to grow and synthesize CGC with different chitin:β-glucan molar ratios under a wide range of temperatures and pH. However, CGC content in the biomass and the volumetric productivity were not significantly affected within the tested pH and temperature ranges. In contrast, the effect of pH and temperature on the polymer's chitin:β-glucan molar ratio was more pronounced. The highest chitin:β-glucan molar ratio (> 14:86) was obtained for the mid-range pH (4.5-5.8) and temperatures (26–33 ºC), while a drastic reduction of chitin to ≤ 6 mol% was observed outside those ranges.

The effect of different concentrations of glycerol byproduct, the presence of toxic or stimulatory compounds and micronutrients content and composition on medium, on CGC production by *K. pastoris*, as well as the effect on yeast cell wall composition, in terms of chitin:β-glucan molar ratio were also evaluated.

K. pastoris yeast was able to use different concentration of glycerol byproduct. At standard conditions, 40g/l of glycerol byproduct, 9.23 g/l of biomass with 15% of CGC content and with a chitin:β-glucan molar composition of 16:84 mol was obtained. Therefore, glycerol byproduct at concentration of 60 g/l and glycerol byproduct at concentration of 40 g/l supplemented with 200 mM of $CaSO_4$ were found to be the better suitable sources of carbon for stimulated cell growth (10.26 and 12.43 g/l, respectively) and also to increased the production level of CGC content on biomass (22 and 24%). Moreover, glycerol byproduct at concentration of 40 g/l supplemented with glucosamine proved good for increased chitin:β-glucan molar ratio in the polymer (23:77 mol%).

The suitability of different substrates such as galactose, lactose and sucrose for the cultivation of the yeast K. *pastoris* and production of CGC were also evaluated. Though K. *pastoris* reached low biomass concentration using galactose, lactose and sucrose as sole carbon sources the CGC content (21-23 wt%) was higher, compared to a standard conditions (15 wt%). The potential of different industrial wastes (sugarcane molasses, cheese whey and spent coffee grounds) or byproducts as substrates for this yeast was also evaluated, to search for alternative substrates to glycerol. K. *pastoris* reached low biomass concentration using these substrates. The best results were achieved with sugarcane molasses, where biomass obtained (17.78 g/l) contained 17% of CGC. Though this value was higher than that (15%) obtained with glycerol byproduct the latter reached a higher cell concentration (33.6 g/l). Thus, the overall amount of CGC may be obtained with glycerol byproduct.

Furthermore, a compromise between the syntheses of CGC with high chitin content and maximize CGC contents on biomass, depending on the final application of this product. The ability of *K. pastoris* to synthesize CGC with different composition, as a result of the modification of cultivation conditions, is remarkable. In view of this, such capability confers the bioprocess a great versatility, enabling to achieve different polymers, which can be used in different applications. Taking into account the chemical composition, combined to the prospective lower production costs from using a low-cost carbon source, CGC has an enormous potential to be used on several industrial applications, namely in cosmetics, pharmaceuticals, agriculture and health.

6.2. *Future Work*

The results obtained in this work may be improved and extended in diverse ways.

Firstly, as described above, *K. pastoris* was able to grow in many substrates including industrial wastes and/or byproducts. However, the wastes tested in this work did not undergo high cellular concentrations, thus compromising the overall CGC productivity of the process. In order to increase biomass growth and, consequently the CGC productivity, operational parameters, such as dissolved oxygen concentration, aeration and stirring rate, can also affect cell growth and CGC production by *K. pastoris* and, hence, their effect should be studied. Lopes et al. (2013)

showed that increasing air pressures may lead to increasing biomass yields of *K. pastoris.* Thus this parameter may be tested and its impact on the volumetric productivity of CGC evaluated. Other bioreactor operation modes (e.g. repeated fed-batch and continuous), as well as other feeding strategies (e.g. pulses) may also be tested and its impact in chitin:β-glucan ratios evaluated. CGC polymers with different composition might possess distinct properties that could be useful for different applications.

The bioprocess can also be optimized in terms of the downstream procedure to yield pure polymers, such as chitin and/or glucans in high yields. In order to separate the chitin from glucans present in CGC several chemical hydrolysis assays could be performed. The protocol for the purification of chitin, using TFA to hydrolyze glucans was already studied by Freimund et al. (2005). However it was found experimentally to have little affinity to break the intramolecular bonds of chitin. The impact of temperature, acid concentration and hydrolysis time may be evaluated in order to obtain high purity chitin and at the lowest possible degradation.

New applications for the CGC should be explored. Due its performance as multifunctional additive, such as excipient (binder, desintegrant, preservative, antioxidant and/or lubricant), recently disclosed on Patent Application WO2013140222 , the potential application of CGC for high-value pharmaceutical, cosmetics and food applications could be evaluated, as well the safety of this biopolymer (by cytotoxic assays) and the biological activity.

Bibliography

Aguilar-Uscanga, A., François, J. (2003). A study of the yeast cell wall composition and structure in response to growth conditions and mode of cultivation. Letters in Applied Microbiology, 37:268–274.

Ahn, W. S., Park, S. J., Lee, S. Y. (2000) Production of Poly(3-Hydroxybutyrate) by fed-batch culture of recombinant *Escherichia coli* with a highly concentrated whey solution. Applied and Environmental Microbiology, 66 (8):3624–3627.

Andrade, V., Neto, B., Fukushima, K., Campos-Takaki, G. (2003) Effect of medium components and time of cultivation on chitin production by *Mucor circinelloides* -A factorial study. Revista Iberoamericana de Micologia, 20:149-153.

Araújo, V., Melo, A., Costa, A., Castro-Gomez, R., Madruga, M., Souza, E., Magnan, M. (2014).. Followed extraction of β-glucan and mannoprotein from spent brewer's yeast (*Saccharomyces uvarum*) and application of the obtained mannoprotein as a stabilizer in mayonnaise. Innovative Food Science & Emerging Technologies, 23:164–170.

Arce, V., Trubey, R., D., Chandler, R., Chandler, C. (2009) Measuring and managing the environmental cost of coffee production in latin america. Conservation and Society, 7(2): 141-144.

Artamonova, S., Sharnina, F. (2013) Applied Biochemical Microbiology, 49:426-431.

Ayoub, M., Abdullah, A. (2012) Critical review on the current scenario and significance of crude glycerol resulting from biodiesel industry towards more sustainable renewableenergy Industry. Renewable and Sustainable Energy Reviews, 16: 2671–2686.

Bai, Y., Peilong, Y., Yaru, W., Pengjun, S., Huiying, L., Kun, M., Bo, W., and Bin, Y. (2009) Phytase production by fermentation of recombinant *Pichia Pastoris* in monosodium glutamate wastewater. World Journal of Microbiology and Biotechnology, 25: 1643–1649.

Berg, J., Tymoczko, L. (2002) Stryer: Biochemistry, WH Freeman and Company, New York 270,465,687.

Bhosale, P, and R V Gadre. (2001) β-Carotene production in sugarcane molasses by a *Rhodotorula Glutinis* mutant. Journal of Industrial Microbiology and Biotechnology, 26: 327–332.

Bowman, M. and Free, S. (2006) The structure and synthesis of the fungal cell wall. Bioassays**,** 28(8):799-808.

Boze, H., Laborde, C., Chemardin, P., Richard, F., Venturin, C., Combarnous, Y. and Moulin, G. (2001) High-level secretory production of recombinant porcine follicle-stimulating hormone by *Pichia pastoris*. Process Biochemistry, 36: 907-913.

Brady, C.P., Shimp, R.L., Miles, A.P., Whitmore, M., Stowers, A.W. (2001) High-level production and purification of P30P2MSP119 an important vaccine antigen for malaria, expressed in the methylotrophic yeast *Pichia pastoris*. Protein Expression and Purification, 23: 468-475.

Brierley, R.A., Bussineau, C., Kosson, R., Melton, A., Siegel, R.S. (1990) Fermentation development of recombinant *Pichia pastoris* expressing the heterologous gene: bovine lysozyme, Ann. N.Y. Academy of Sciences, 589: 350-362.

Bulik, D., Olczak,M., Lucero, H., Osmond, B., Robbins, P., Specht, C. (2003) Chitin synthesis in *Saccharomyces cerevisiae* in response to supplementation of growth medium with glucosamine and cell wall stress. Eucaryotic Cell, 2(5):886–900.

Çalik,P., Bayraktar, E., Inankur, B., Soyaslan, E., Sahin, M., Taspınar, H., Açık, E., Yilmaz, R., Ozdamard, T.; J. (2010) Chemical Technology Biotechnology, 85: 1628–1635.

Celik E, Ozbay N, Oktar N, Calik P. (2008) Use of biodiesel byproduct crude glycerol as the carbon source for fermentation processes by recombinant *Pichia pastoris*. Industrial Engineering Chemical Research, 47(9):2985–90.

Cereghino, J. and Cregg, J. (2000) Heterologous protein expression in the methylotrophic yeast *Pichia Pastoris*. FEMS Microbiology Reviews, 24: 45–66.

Cereghino, G., Cereghino, J.L., Ilgen, C., Cregg, J.M. (2002) Production of recombinant proteins in fermenter cultures of the yeast *Pichia pastoris*. Current Opinion in Biotechnology, 13: 329-332.

Cervera MF, Heinamaki J, Rasanen M, Maunu SL, Karjalainen M, Nieto Acosta OM. (2004) Solid-state characterization of chitosans derived from lobster chitin. Carbohydrate Polymers, 58:401–8.

Chauhan AK, Arora D, Khanna N., 1999. A novel feeding strategy for enhanced protein production by fed-batch fermentation in recombinant *Pichia pastoris*. Process Biochemistry, 34(2):139–45.

Chiruvolu, V., Eskridge, K., Cregg, J., Meagher, M. (1998) Effects of glycerol concentration and pH on growth of recombinant *Pichia pastoris* yeast. Applied Biochemical and Biotechnology, 75:163-173.

Cregg, J.M., Vedvick, T.S. and Raschke W.C. (1993) Recent advances in the expression of foreign genes in *Pichia pastoris*. Bio/Technology. 11:905-910.

Cregg J.M., Cereghino, J.,L., Shi, J., Higgins, D.,R. (2000) Recombinant Protein Expression in Pichia pastoris. Molecular. Biotechnology. 16(1), 23–52.

Cruz GM. (1983) Resíduos de cultura e indústria. Informe Agropecuário, 9:32–7.

Cos, O., Ramón,R., Montesinos, J.,Valero, F. (2006) Operational strategies, monitoring and control of heterologous protein production in the methylotrophic yeast *Pichia pastoris* under different promoters: A review. Microbial Cell Factories:5: 17.

Couderc, R.; Baratti J. (1980) Oxidation of methanol by the yeast *Pichia Pastoris*. Purification and properties of the alcohol oxidase agricultural and biological chemistry, 44 (10): 2279–2289.

Cui, X, and M Ellison. (2012) Effects of biodiesel waste glycerol on the growth characteristics of *Pichia pastoris* genetically modified to produce spidroin. International Journal of ChemTech Research, 4 (2): 713–719.

Cunha, A, Clemente,J, Gomes,R., Pinto,F., Thomaz, M., Miranda, S., Pinto, R., Moosmayer, D., Donner, P., Carrondo, M. (2004) Methanol induction optimization for scFv antibody fragment production in *Pichia pastoris*; Biotechnology and Bioengineering, 86(4):458-67.

D'Anjou, M. and A. J. Daugulis. (2000) Mixed-feed exponential feeding for fed-batch culture of recombinant methylotrophic yeast. Biotechnology Letters, 22: 341-346.

D'Anjou, M. and Daugulis, A. (2001) A rational approach to improving productivity in recombinant *Pichia pastoris* fermentation. Biotechnology and Bioengineering, 5;72(1):1-11.

Davenport K., Sohaskey, M., Kamada, Y., Levin, D., Gustin, M. (1995) A second osmosensing signal transduction pathway in yeast. Hypotonic shock activates the PKC1 protein kinase-regulated cell integrity pathway. Journal of Biological Chemistry, 270(50):30157–30161.

De Nobel, J. G., Klis, F. M., Priem, J., Munnik, T., and Van Den Ende, H. (1990) The glucanase-soluble mannoproteins limit cell wall porosity in Saccharomyces cerevisia Yeast, 6: 491–499.

Donati, E., Sand, W. (2007) Microbial Processing of Metal Sulfides. Netherlands. Springer.

Du, L., Zhang, X., Wang, C., Xiao D. (2012) Preparation of water soluble yeast glucan by four kinds of solubilizing processes; Engineering, 5: 184-188.

Dutta, P K, Joydeep Dutta, and V S Tripathi. (2004) Chitin and Chitosan : Chemistry, properties and applications. Journal of Scientific & Industrial Research, 63: 20–31.

EFSA Panel on Dietetic Products (2010) Nutrition and Allergies (NDA). Scientific opinion on the safety of "chitin–glucan" as a novel food ingredient. EFSA J, 7:1687.

Einbu, A., Vårum, K. (2008) Characterization of chitin and its hydrolysis to GlcNAc and GlcN. Biomacromolecules, 9: 1870–1875.

Farinha, I., Duarte, P., Pimente, A., Plotnikova, E., Chagas, B., Mafra, L., Grandfils, C., Freitas, F., Fortunato, E., Reis, M. (2015) Chitin-glucan complex production by *Komagataella pastoris*: downstream optimization and product characterization. Carbohidrate Polymers, 130: 455-464.

Feofilova, E., Tereshina, V., and Memorskaya, A. (1995) Chitin of mycelial fungi: isolation and identification methods and physico-chemical properties. Mikrobiologiya, 64:27.

Feofilova,E. (2002) Chitin and Chitosan: Synthesis, properties and applications, Ed. by K. G. Skryabin,G. A. Vikhoreva, and V. P. Varlamov, p. 368.

Feofilova, E P, Nemtsev, D V, Tereshina, V M and Memorskaya A S. (2006) Developmental change of the composition and content of the chitin – glucan complex in the fungus *Aspergillus niger*. Applied Biochemistry and Microbiology, 42: 624–628.

Ferreira, A, Ataíde, F., Von Stosch, M., Dias, J., Clemente, J., Cunha, A., Oliveira, R. (2012) Application of adaptive DO-stat feeding control to *Pichia pastoris* X33 cultures expressing a single chain antibody fragment (scFv). Bioprocess Biosystems Engineering, 35(9):1603-14.

Files, D., Ogawa,M., Scaman, C., Baldwin, S. (2001) A *Pichia pastoris f*ermentation process for producing high-levels of recombinant human cystatin-C. Enzyme and Microbial Technology, 29: 335–340.

Fischer, P.W.F., Giroux, A., L'Abbe, M.R. (1984) Effect of zinc supplementation on copper excretion and retention in men. American Journal Clinical Nutrition, 40:743-6.

Fleet, G., Manners, D. (1976) Isolation and composition of an alkali-soluble glucan from the cell walls of *Saccharomyces cerevisiae*. Journal General Microbiology, 94: 180-192.

Fleet, G. (1991) Cell walls. In Rose AH, Harrison JS. ed; The Yeasts. 2nd edn. Vol. 4. London: Academic Press,199–277.

Freitas, F., Roca, C., Silva, F., Reis, M., Frinha, I., Chagas, B., Oliveira, R. (2013) Natural biocomposite powder prepared from *Pichia pastoris* biomass, method of preparation and its use as excipient, WO2013140222.

Freimund, S., Janett, S., Arrigoni, E., Amadò, R. (2005) Optimised quantification method for yeast-derived 1,3-β-D-glucan and α-D-mannan. European Food Research Technology, 220:101–105.

Garcia, R., Bermejo, C., Grau, C., Perez, R., Rodriguez-Pena, J., Francois, J., Nombela, C., Arroyo, J. (2004) The global transcriptional response to transient cell wall damage in *Saccharomyces cerevisiae* and its regulation by the cell integrity signaling pathway. Journal of Biologycal Chemistry, 279: 15183–15195.

Gancedo, M. (1998) Yeast carbon catabolite repression. Microbiology and Molecular Biology Reviews, 62(2): 334.

Gasser, B., Maurer, M., Rautio, J., Sauer, M., Bhattacharyya, A., Saloheimo, M., Penttilä, M., Mattanovich, D. (2007) Monitoring of transcriptional regulation in *Pichia pastoris* under protein production conditions. BMC Genomics, 8:179.

Gautier, S.; Xhauflaire-Uhoda, E.; Gonry, P.; Pierard, G. (2008) Chitin–glucan, a natural cell scaffold for skin moisturization and rejuvenation; International Journal of Cosmetic Science, 30: 459–469.

Gerde, J. A., M. Montalbo Lomboy, L. Yao, D. Grewell, and T. Wang. (2012) Evaluation of microalgae cell disruption by ultrasonic treatment. Bioresources Technology, 125:175-181.

Ghosalkar, A., V. Sahai, and A. Srivastava. (2008) Optimization of chemically defined medium for recombinant *Pichia pastoris* for biomass production. Bioresources Technology, 99: 7906-7910.

González-Barroso, M., Ledesma, A., Lepper, S., P´erez-Mag´an, E., Zaragoza, P. and Rial, E. (2006) Isolation and bioenergetic characterization of mitochondria from *Pichia pastoris*. Yeast, 23: 307–313.

Guimarães, P., Teixeira, M., Domingues, L. (2010) Fermentation of lactose to bio-ethanol by yeasts as part of integrated solutions for the valorization of cheese whey. Biotechnology Advances, 28: 375–384.

Ha, C., Lim, K., Kim, Y., Lim, S., Kim, C., Chang, S. (2002) Analysis of alkali-soluble glucan produced by *Saccharomyces cerevisiae* wild-type and mutants. Applied Microbiology Biotechnology, 58:370–377.

Hsieh, J., Wu, H., Wei, Y. (2007) Determination and kinetics of producing glucosamine using fungi. Biotechnology Progress, 23: 1009-1016.

Hughes, S., López-Núñez, J., Jones, M., Moser, B., Cox, E., Lindquist, M., Galindo-Leva, L., Riaño-Herrera, N., Rodriguez-Valencia, N., Gast, F., Cedeño, D., Tasaki, K., Brown, R., Darzins, A., Brunner, L. (2014) Sustainable conversion of coffee and other crop wastes to biofuels and bioproducts using coupled biochemical and thermochemical processes in a multi-stage biorefinery concept. Applied Microbiology Biotechnology, 98:8413–8431.

Inan, M., Chiruvolu, V., Eskridge, K. M., Vlasuk, G. P., Dickerson, K., Brown, S. and Meagher, M. M. (1999) Optimization of temperature-glycerol-pH conditions for a fed-batch fermentation process for recombinant hookworm (*Ancylostoma caninum*) anticoagulant peptide (AcAP-5) production by *Pichia pastoris*. Enzyme Microbiology Technology, 24(7): 438-445.

Inan, M., Meagher, M.M. (2001-b) Non-repressing carbon sources for alcohol oxidase (AOX1) promoter of *Pichia pastoris*. Journal of Bioscience and Bioengineering, 92: 585-589.

Ivshin V.P., S.D. Artamonova, T.N. Ivshina and F.F. Sharnina. (2007) Methods for isolation of chitin-glucan complex from higher fungi native biomass. Polymer Science, 49: 305–310.

Ivshina TN, Artamonova SD, Ivshin VP, Sharnina FF. (2009) Isolation of chitin–glucan complex from the fruiting bodies of Mycothallus. Applied Biochemestry Microbiology, 45(3):313–8.

Jahic M, Rotticci-Mulder JC, Martinelle M, Hult K, Enfors SO. (2002) Modeling of growth and energy metabolism of *Pichia pastoris* producing a fusion protein. Bioprocess Biosystems Engineering, 24:385-93.

Jayakody,L.,Tsuge,K., Suzuki,A., Shimoi, H., and Kitagaki, H. (2013) Identification of the sulphate ion as one of the key components of yeast spoilage of a sports drink through genome-wide expression analysis. Journal General Applied Microbiology, 59: 227–238.

Johnston, I. (1965) The Composition of the cell wall of *Aspergillius niger*. Biochemestry Journal, 96: 651.

Jungo, C., Rerat, C., Marison, I.W., Von Stockar, U. (2006) Quantitative caracterization of the regulation of the synthesis of alcohol oxidase and of the expression of recombinant avidin in a *Pichia pastoris* Mut+ strain. Enzyme Microbiology Technology, 39 (4): 936–944.

Jungo, C., Marison, I. W. and von Stockar, U. (2007) Mixed feeds of glycerol and methanol can improve the performance of *Pichia pastoris* cultures: A quantitative study based on concentration gradients in transient continuous cultures. Journal of Biotechnology in press, 128:824-837.

Kaya, M., Erdogan, S., Mol, A., & Baran, T. (2015). Comparison of chitin structures isolated from seven *Orthoptera species*. International journal of biological macromolecules, *72*: 797-805.

Kamada, Y., Jung, U., Piotrowski, J., Levin, D. (1995) The protein Kinase C-activated kinase pathway of *Saccharomyces cerevisiae* mediates a novel aspect of heat shock response. Genes & Development, 9:1559–1571.

Kanarskaya, Z. (2000) Extended abstract of candidate's dissertation in technical sciences.

Kapteyn JC, Van Den Ende H, Klis FM. (1999) The contribution of cell wall proteins to the organization of the yeast cell wall. Biochimica et Biophysica Acta, 1426:373–383.

Ketela, Troy, Robin Green, and Howard Bussey. (1999) *Saccharomyces Cerevisiae* Mid2p is a potential cell wall stress sensor and upstream activator of the PKC1-MPK1 cell integrity pathway. Journal of Bacteriology, 181: 3330–3340.

Khanafari A, Marandi R, Sanatei S. (2008) Recovery of chitin and chitosan from shrimp waste by chemical and microbial methods. Iranian Journal of Environment Health, Science and Engineering, 5(1):19–24.

Kittur FS, Prashanth KVH, Sankar KU, Tharanathan RN. (2002) Characterization of chitin, chitosan and their carboxymethyl derivatives by differential scanning calorimetry. Carbohydrate Polymers, 49(2):185–93.

Klis FM. (1994) Review: cell wall assembly in yeast. Yeast, 10:851–869.

Klis, F., Boorsma, A. ,Groot, P. (2005) Cell wall construction in *Saccharomyces cerevisiae.* Yeast, 23:185–202.

Knorr, D. (1991) Recovery and utilization of chitin and chitosan in food processing waste management. Food Technology, 45 (1): 114–122.

Kollar, R., Petra´kova, E., Ashwell, G., Robbins, P. and Cabib, E. (1995) Architecture of the yeast cell wall – The linkage between chitin and β-(1-3)-glucan. The Journal of Biological Chemistry, 270(3):1170-1178.

Kollar, R, Reinhold BB, Petrakova E, Yeh HJ, Ashwell G, Drgonova J. (1997) Architecture of the yeast cell wall. β-(1,6)-glucan interconnects mannoprotein, β-(1,3)-glucan, and chitin. Journal of Biological Chemistry, 272:17762–17775.

Koller, M; Braunegg, G; Hesse, P; Kutschera, C; Atlic, A; Bona, R. (2007) Potential of various archae- and eubacterial strains as industrial polyhydroxyalkanoate producers from whey. Macromolecular Bioscience, 7:218-226.

Koller, M., Bona, R., Chiellini, E., Fernandes, E. G., Horvat, P., Kutschera, C., Hesse, P., Braunegg, G. (2008) Polyhydroxyalkanoate production from whey by *Pseudomonas hydrogenovora*. Bioresource Technology, 99:4854– 4863.

Kotlyar, M., 2001. Extended abstract of candidate's dissertation in technical Sciences.

Kuranda, K., Leberre, V., Sokol S., Palamarczyk, G., François J. (2006) Investigating the caffeine effects in the yeast *Saccharomyces cerevisiae* brings new insights into the connection between TOR, PKC and Ras/cAMP signalling pathways. Molecular Microbiology, 61(5):1147-66.

Landecker, M. (1996) Fundamentals of the fungi. New Jersey: Prentice Hall.

Lee, S.Y., Middelberg, A., Lee Y.(1997) Poly(3-hydroxybutyrate) production from whey using recombinant *Escherichia coli*. Biotechnology Letters, 19 (10):1033–1035.

Leeson S, Summers J. (2000) Nutrición aviar comercial. LePrint Club Express Ltda Bogotá, Colombia.

Li, Z., Xiong, F., Lin, Q., D'Anjou, M., Daugulis, A., Yang, D. and Hew, C.(2001) Low-temperature increases the yield of biologically active herring antifreeze protein in *Pichia pastoris.* Protein Expression and Purification, 21: 438-445.

Li, A., Crimmins, D. L., Luo, Q., Hartupee, J., Landt, Y., Ladenson, J. H., Wilson, D., Anant, S. and Dieckgraefe, B. (2003) Expression of a novel regenerating gene product, Reg IV, by high density fermentation in *Pichia pastoris*: production, purification, and characterization. Protein Expression and Purification, 31(2):197-206.

Lipke, Peter N, and Ovalle, R. (1998) Cell wall architecture in yeast : new structure and new challenges. Journal of Bacteriology, 180: 3735–3740.

Lopes M, Mota M, Belo I. (2013) Batch and fed-batch growth of *Pichia pastoris* under increased air pressure. Bioprocess and Biosystems Engineering, 36:1267–1275.

Lundstedt, T., Seifert, E., Abramo, L., Thelin, B., Nyström, A., Pettersen, J., Bergman, R. (1998) Experimental desing and optimization. Chemometrics and Intelligent Laboratory Systems, 42: 3-40.

Macauley-Patrick, S., Fazenda, M., McNeil, B., Harvey, L. (2005) Heterologous protein production using the *Pichia pastoris* expression system. Yeast, 22:249–270.

Magnelli, P., Cipollo, J., Abeijon, C. (2002) A Refined method for the determination of *Saccharomyces cerevisiae* cell wall composition and β-1,6-glucan fine structure. Analytical Biochemistry, 301(1):136–150.

Martin, H., Rodriguez-Pachon, J., Ruiz, C., Nombela, C., Molina, M. (2000) Regulatory mechanisms for modulation of signaling through the cell integrity Slt2-mediated pathway in *Saccharomyces cerevisiae.* Journal of Biology Chemistry, 275:1511–1519.

Meichik, N., Vorob'ev, D. (2012) Chitin-glucan complex in cell walls of the *Peltigera aphthosa* lichen. Applied Biochemistry and Microbiology, 48:307–311.

Mussatto SI, Machado EMS, Martins S, Teixeira J. (2011) Production, composition and application of coffee and its industrial residues. Food and Bioprocess Technology,4:661–72.

Mussato S., Machado E., Martins S., Teixeira A. (2011a) Production, composition , and application of coffee and its industrial residues. Food and Bioprocess Technology, 4:661–672.

Mussatto, I., Carneiro, L., Silva, J., Roberto, I.,Teixeira, J. (2011b) A Study on chemical constituents and sugars extraction from spent coffee grounds. Carbohydrate Polymers, 83: 368–374.

Mussatto S, Machado, E., Carneiro, L., Teixeira, J. (2012) Sugars metabolism and ethanol production by different yeast strains from coffee industry wastes hydrolysates. Applied Energy, 92:763–768.

Muzzarelli, R., Muzzarelli, C., Terbojevich, M. (1997) Chitin chemistry, upgrading a renewable resource. Carbohydrates in Europe, 19:10-17.

Nguyen TH, Fleet GH, Rogers PL. (1998) Composition of the cell walls of several yeast species. Applied Microbiology and Biotechnology, 50(2):206-12.

Nevoigt, E. and Stahl, U. (1997) Osmoregulation and glycerol metabolism in the yeast *Shacaromyces cereviasae*. FEMS Microbiology Reviews, 21(3):231-41.

Nielsen, J., Villadsen, J. (1994) Bioreaction Engineering Principles, Second Edition, Plenum Press, New York.

Novak, M., Synytsya, A., Gedeon, O., Slepicka, P., Prochazka, V., Blahovec, J., Hejlova, A., & Copikova, J. (2012). Yeast β(1-3),(1-6)-D-glucan films: Preparation and characterization of some structural and physical properties. Carbohydrate Polymers, *87*(4): 2496-2504.

Olbrich H. (2006) The molasses. Biotechnologie-Kempe GmbH, Germany.

Oliveira R, Clemente J, Cunha A., Carrondo M..(2005) Adaptive dissolved oxygen control through the glycerol feeding in a recombinant *Pichia pastoris* cultivation in conditions of oxygen transfer limitation. Journal of Biotechnology, 116(1):35–50.

Orlean, P. (1997) Biogenesis of yeast wall and surface components. In Molecular and Cellular Biology of the Yeast *Saccharomyces cerevisiae.* Cell Cycle and Biology (Pringle, J., Broach, J., and Jones, E., Eds.), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. Vol 3: 229–326.

Osumi M. (1998) The ultrastructure of yeast: cell wall structure and formation. Micron, 29(2/3):207-233.

Otero, M., Vasallo, M., Verdecia, O., Fernandez, V, Betancourt, D. (1996) A Process for the complete fractionation of baker's yeast. Journal of Chemical Technology and Biotechnology, 67:67-71.

Pachauri, N., He, B. (2006) Value-added utilization of crude glycerol from biodiesel production: a survey of current research activities.ASABE Paper No. 066223. St. Joseph, Mich.: ASABE.

Park BK and Kim MM. (2010) Applications of chitin and its derivatives in biological medicine. International Journal of Molecular Sciences, 11(12):5152–64.

Percot, A., Viton, C., Domard, A., 2003. Optimization of chitin extraction from shrimp shells. Biomacromolecules, 4:12-18.

Pestov, A., Drachuk, S., Koryakova, O., Yatluk, Y. (2009) Isolation and characterization of chitin-glucan complexes from the Mycothallus of fungi belonging to *Russula* genus. Chemistry for Sustainable development, 17:281-287.

Phaff, Herman J. (2001) Yeasts. In encyclopedia of life sciences, 1–11.

Pillai R., Redmond M., and Roding J. (2005) Anti-wrinkle therapy: significant new findings in the non-invasive cosmetic treatment of skin wrinkles with beta-glucans. International Journal of Cosmetic Science, 27:292.

Plantz, B. A., K. Nickerson, S. D. Kachman, and V. L. Schlegel. (2007) Evaluation of metals in a defined medium for *Pichia pastoris* expressing recombinant beta-galactosidase. Biotechnology Progress, 23:687-692.

Puligundla, P., Smogrovicova, D., Obulam, V.S.R., Ko, S. (2011) Very high gravity (VHG) ethanolic brewing and fermentation: a research update. Journal of Industrial Microbiology and Biotechnology, 38:1133–1144.

Prashanth KVH, Kittur FS, Tharanathan RN. (2002) Solid state structure of chitosan prepared under different N-deacetylating conditions. Carbohydrate Polymers, 50(1):27–33.

Ramakrishnan, S., and Hartley, B. (1993) Fermentation of lactose by yeast cells secreting recombinant fungal lactase. Applied and Environmental Microbiology, 59(12): 4230–4235.

Reis, M.A.M., Oliveira, R., Freitas, F., Alves, V.D. (2011) Fucose-containing bacterial biopolymer, WO 2011/073874 A2.

Reis, M., Oliveira, R., Freitas, F., Chagas, C., Cruz, A., Cunha, A., Clemente, J. (2010) Process for the co-production of chitin, its derivatives and polymers containing glucose, mannose and/or galactose, by the fermentation of the yeast *Pichia pastoris*, WO2010013174.

Ren, H. T., Yuan, J. Q., Bellgardt, K. H. (2003) Macrokinetic model for methylotrophic *Pichia pastoris* based on stoichiometric balance. Journal of Biotechnology, 106:53-68.

Rinaudo M. (2006) Chitin and chitosan: properties and applications. Progress in Polymer Science, 31(7):603–32.

Roca, Christophe, Chagas, B., Farinha, I., Freitas, F., Mafra, L., Aguiar, F., Oliveira, R., and Reis, M. (2012) Production of yeast chitin–glucan complex from biodiesel industry byproduct. Process Biochemistry, 47 (11): 1670–1675.

Roncero, C., Durán, A. (1985) Effect of calcofluor white and congo red on fungal cell wall morphogenesis: in vivo activation of chitin polymerization. Journal of Bacteriology, 163(3):1180-1185.

Ruiz - Herrera, J. (1978) The distribution and quantitative importance of chitin in fungi. In Muzzarelli, R. A. A. and J. Priser (eds.). Chitin and Chitosan, Proc. First Internal. Conf. MIT, USA. pp. 11 – 21.

Russo, O., Simonen, M., Uimari, A., Teesalu, T., Makarow, M. (1993) Dual regulation by heat and nutrient stress of the yeast *HSP150* gene encoding a secretory glycoprotein. Molecular and General Genetics, 239: 273-280.

Saltukoglu, A, Slaughter, J. (1983) The effect of magnesium and calcium on yeast growth. Journal of the Institute of Brewing, 89:81-83.

Serio, M., Aramo, M., Alteriis, E., Tesser, R. and SantacesariaE. (2003) Quantitative analysis of the key factors affecting yeast growth. Industrial & Engineering Chemical Reserach,42 (21):5109–5116.

Scragg, A.H. (1988) Biotechnology for engineers: Biological systems in technological processes. E. Horwood. Chichester, West Sussex, England and New York.

Shahidan, N H, R N Z A Rahman, T C Leow, M Rosfarizan, and M Basri. (2011) The effect of carbon sources on the expression level of thermostable L2 lipase in *Pichia pastoris*. African Journal of Biotechnology, 10 (12): 13528–13535.

Shang F, Wen S, Wang X, Tan T. (2006) High-cell-density fermentation for ergosterol production by *Saccharomyces cerevisiae*. Journal of Bioscience and Bioengineering, 101(1):38-41.

Shi, X., Karkut, T., Chamankhah, M., Alting-Mees, M., Hemmingsen, S. M. and Hegedus, D. (2003) Optimal conditions for the expression of a single-chain antibody (scFv) gene in *Pichia pastoris*. Protein Expression and Purification, 28: 321-330.

Silva, M., Nebra, S., Machado,M., Sanchez, C. (1998) The use of biomass residues in the Brazilian soluble coffee industry. Biomass Bioenergy, 14:457–67.

Siso, M. (1996) The biotechnological utilization of cheese whey: a review. Bioresource Technology, 57: 1-11.

Smirnou, Dzianis, Martin Krcmar, and Eva Prochazkova. (2011) Chitin-glucan complex production by *Schizophyllum commune* submerged cultivation. Polish Journal of Microbiology, 60 (3): 223–228.

Smits, J., Rinzema, A., Tramper, T., Sonsbeek, H., Hage, J., Kaynak, A., Knol, W. (1998) *The* influence of temperature on kinetics in solid-state fermentation. Enzyme and Microbial Technology, 22: 50–57.

Solá, A., Maaheimo, H., Ylo len, K., Ferrer, P. & Szyperski, T. (2004) Amino acid biosynthesis and metabolic flux profiling of *Pichia pastoris*. European Journal of Biochemistry, 271:2462–2470.

Soyaslan, E., Çalik,P. (2011) Enhanced recombinant human erythropoietin production by *Pichia pastoris* in methanol fed-batch/sorbitol batch fermentation through pH optimization. Biochemical Engineering Journal, 55:59–65.

Spevacek J, Brus J. (2008) Solid-state NMR studies of polysaccharide systems. Macromolecular Symposia, 265:69–76.

Sreekrishna K., Tschopp J., Fuke M. (1987) Invertase gene (SUC2) of *Saccharomyces cerevisiae* as a dominant marker for transformation of *Pichia pastoris*. Gene, 59(1**):**115–125.

Sreekrishna, K., Brankamp, R., Kropp, K., Blankenship, D., Tsay, J., Smith, P., Wierschke, J., Subramaniam, A. and Birkenberger, L. (1997) Strategies for optimal synthesis and secretion of heterologous proteins in the methylotrophic yeast *Pichia pastoris*. Gene, 190:55-62.

Stehlik- Tomas, V., Zetic, V., Stanzer, D., Grba, S., Vahcic, N. (2004) Zn, Cu and Mn Enrichment in *S. cerevisiae,* Food Technology and Biotechnology, 42 (2):115–120.

Stoscheck CM. (1990) Quantitation of protein. Methods in Enzymology, 182:50–69.

Stratton, J., V. Chiruvolu, M. Meagher. (1998) High cell density fermentation. Biotechnology Advances, 103:107-120.

Su, C., Sun, C., Juan, S., Hu, C., Ket, W., Sheut, M. (1997) Fungal mycelia as the source of chitin and polysaccharides and their applications as skin substitutes. Biomaterials, 16:1169-1174.

Sugawara T., Takahashi S., Osumi M., Ohn N.(2004) Refinement of the structures of cell-wall glucans of *Schizosachharomyces pombe* by chemical modification and NMR spectroscopy. Carbohydrate Research, 339: 2255–2265.

Synowiecki J, Al-Khateeb N. (2003) Production, properties and some new applications of chitin and its derivates. Critical Reviews in Food Science and Nutrition, 43(2): 145-171.

Tarabukina EB, Kalinina NA, Adamov AV, Petrova VA, Nud'ga LA, Klenin SI. (2005) Molecular characteristics and supramolecular organization of chitin–glucan complexes in solutions. Polymer Science Series A, 47(5):462–8.

Thorpe, E., D'Anjou, M., Daugulis, A. (1999) Sorbitol as a non-repressing carbon source for fed-batch fermentation of recombinant *Pichia pastoris*. Biotechnology Letters, 21: 669–672.

Tokimoto T, Kawasaki N, Nakamura T, Akutagawa J, Tanada S. (2005) Removal of lead ions in drinking water by coffee grounds as vegetable biomass. Journal of Colloid and Interface Science, 281:56–61.

Tomita, M., Iwata, S., Yamamoto, S. (1996) Alternation in cell wall chitin of *Zygosaccharomyces rouxii*. Journal of Fermentation and Bioengineering, 81:171-173.

Ul-Haq I, Ali S, Iqbal J. (2002) Influence of cultivation conditions on citrate production by *Aspergillus niger* in a semi-pilot-scale plant. Folia Microbiologica, 47(5):511-515.

Veana, F., Martínez-Hernández, J., Aguilar, C., Rodríguez-Herrera, R., Michelena, G. (2014) Utilization of molasses and sugar cane bagasse for production of fungal invertase in solid state fermentation using *Aspergillus niger* GH1. Brazilian Journal of Microbiology, 45(2):373-377.

Versali, M.-F., Clerisse, F., Bruyere, J.-M. and Gautier, S. (2003) Cell Wall Derivatives from Biomass and Preparation Thereof, WO03/068824.

Xie, J., Zhou, Q., Du, P., Gan, R., Ye, Q. (2005) Use of different carbon sources in cultivation of recombinant *Pichia pastoris* for angiostatin production. Enzyme and Microbial Technology, 36: 210-216.

Yamada, Y., Matsuda, M., Maeda, K., Mikata, K. (1995) The phylogenetic relationships of methanol assimilating yeasts based on the partial sequences of 18S and 26S ribosomal RNAs: the proposal of *Komagataella* gen. nov. (Saccharomycetaceae). Bioscience, Biotechnology and Biochemestry, 59:439-444.

Yen MT, Mau JL. (2007) Physico-chemical characterization of fungal chitosan from shiitake stipes. LWT, 40:472–9.

Yu, X., Dong, T., Zheng, Y., Miao, C., Chen, S. (2014) Investigations on cell disruption of oleaginous microorganisms: Hydrochloric acid digestion is an effective method for lipid extraction. European Journal of Lipid Science and Technology, 117(5):730-737.

Walker, G.M. (1998) Yeast: physiology and biotechnology. John Wiley & Sons Inc., New York.

Wedler, F. (1994) Biochemical and nutritional role of manganese: an overview. In: *Manganese in Health and Disease*, D. J. Klimis-Tavantzis (Ed.), CRC Press, Inc., Boca Raton; pp. 1–38.

Wegner, E.H. (1983) Biochemical conversions by yeast fermentation at high-cell densities. U.S. Patent 4, 414,329. US, Phillips Petroleum Company.

Weinhandl, K., Winkler, M., Glieder A. and Camattari, A. (2014) Carbon source dependent promoters in yeasts. Microbial Cell Factories, 13:5.

Wu, J., Wang, S., Fu, W. (2012) Lower temperature cultures enlarge the effects of *Vitreoscilla* hemoglobin expression on recombinant *Pichia pastoris*. International Journal of Molecular Science, 13:13212-13226.

Zhang, H; Obias, V; Gonyer, K; Dennis, D. (1994) Production of polyhydroxyalkanoates in sucrose-utilizing recombinant *Escherichia coli* and *Klebsiella* strains. Applied and Environmental Microbiology, 60:1198-1205.

Zlotnik, H., Fernandez, M. P., Bowers, B., and Cabib, E. (1984) *Saccharomyces cerevisiae* mannoproteins form an external cell wall layer that determine wall porosity. Journal of Bacteriology, 159:1018–1026.

Zydney, A. L. (1998) Protein separation using membrane filtration: new opportunities for whey fractionation. International Dairy Journal, 8:243–250.